EARLY LIFE OVERFEEDING AND ITS EFFECTS ON HYPOTHALAMIC-PITUITARY-ADRENAL AND SYMPATHO-ADRENAL-MEDULLARY AXES

A thesis submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy** (Laboratory and Clinical Sciences)

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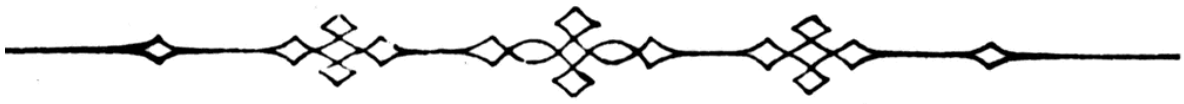

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed; I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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Thesis Contributions

I would like to thank all my colleagues that contributed to finish this thesis.

This thesis is conceived and designed by Guohui Cai and A. Prof. Sarah J. Spencer. Guohui Cai and A. Prof. Sarah J. Spencer also conducted statistical analyses. Guohui Cai, Ilvana Ziko, and A. Prof. Sarah J. Spencer ran the animal studies and collected samples for Chapter 2 and 3 studies. Guohui Cai, Ilvana Ziko, and A. Prof. Sarah J. Spencer helped collect tissue samples for Chapter 4 and 5 studies. Dr. Luba Sominsky helped to finish the *in vitro* studies of adrenal corticosterone responses that were not included in this thesis but that we included in the paper published from Chapter 2. She also helped conduct the liver cytokines assay in Chapter 4. Ilvana Ziko helped to count the numbers of hypothalamic microglia that were not included in this thesis but that we included in the paper published from Chapter 4.

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Presentations

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Glossary of Abbreviations

1

11β-HSD	11β-hydroxysteroid dehydrogenase
3D	3 days
3W	3 weeks

A

α	Alpha
ABS	Australian Bureau of statistics
ACTH	Adrenocorticotropic hormone
AIHW	Australian Institute of Health and Welfare
ANOVA	Analysis of variance
AVP	Arginine vasopressin

B

β	Beta
BMI	Body mass index

C

CD3	Cluster of differentiation 3
cDNA	Complementary deoxyribonucleic acid
c-Fos	Cellular Fos
CH	Chow diet
CL	Contral litter

COX-2	Cyclooxygenase-2
CRH	Corticotrophin-releasing hormone
CTB	Cholera toxin-B chain (CTB)
CV	Coefficient of variation

E

ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunoSorbent assay

F

Fos	A proto-oncogene that is the human homolog of the retroviral oncogene v-fos

G

γ	Gamma
GC	Glucocorticoid
GC	Glucocorticoids
GPO	Glycerol-3-phosphate oxidase
GR	Glucocorticoids receptor
GTT	Glucose tolerance test

H

HDL	high density lipoprotein
HFD	High fat diet
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
hs-CRP	High-sensitivity c-reactive protein

I

i.p.	Intraperitoneal
iAUC	Incremental area under the curve
icv	Intracerebroventricular
IκB	Inhibitory factor κB
IKK	IκB kinase
IKKβ	IKK subunit β
IL	Interleukin
IL-n	Interleukin-n

K

κ	Kappa

L

LDL	low density lipoprotein
LPS	Lipopolysaccharide

M

MC2R	Melanocortin 2 receptor
MC	Mineralocorticoid
MCs	Mineralocorticoids
mgPVN	Magnocellular PVN
mpPVN	Medial parvocellular PVN
MR	Mineralocorticoid receptor
MRAP	Melanocortin 2 receptor accessory protein
mRNA	Messenger eibonucleic acid
MyD88	Myeloid differentiation primary response gene

N

NFκB	Nuclear factor κB
NTS	Nucleus of the solitary tract
MW	Molecular weight

O

OVLT	Lamina terminalis

P

P	Postnatal day
p	Probability value
PAP	Phenol + aminophenazone
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PHA-L	Phageolus vulgaris leucoagglutinin
POMC	Pro-opiomelanocortin
PVDF	Polyvinylidene difluoride
PVN	Paraventricular nucleus of the hypothalamus

R

RMIT	Royal Melbourne Institute of Technology
RNA	Ribonucleic acid
rt-PCR	Quantitative real time polymerase chain reaction

S

s.c.	Subcutaneous

SAM	Sympatho-adrenal--medullary
SEM	Standard error of the mean
SL	Small litter
SOCS3	Suppressor of cytokine signalling 3

T

TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TTC	Tetanus toxin-fragment

V

vBNST	Ventral bed nucleus of the stria terminalis
VLM	Ventral medulla
VMPOA	Ventromedial preoptic area

Z

ζ	Zeta

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Abstract

Introduction: Overweight and obesity is becoming more and more common in Australia. The percentage of obesity in children has increased every year. Obese children in a particular are considered more likely to become obese in adulthood, which leads to a higher risk of developing short and long-term health problems and behaviours, such as Type 2 diabetes, cardiovascular disease and the response to stress. **Hypothesis:** Neonatal nutrition programs development of the hypothalamic-pituitary-adrenal (HPA) axis and sympatho-adrenal--medullary (SAM) axis. **Aim: 1.** To determine how the HPA axis is altered in male rats by neonatal overfeeding. **2.** To determine how the HPA axis is altered in female rats by neonatal overfeeding. **3.** To investigate if neonatal overfeeding makes an animal more susceptible to the effects of a high fat diet. **4.** To determine how the adrenal catecholamine response to Lipopolysaccharide (LPS) is altered by neonatal overfeeding. **Results:** The neonatally overfed male and female rats are heavier than their normal weight counterparts throughout the juvenile phase and into adulthood. The male and female rats have hyper-responsive HPA axes to LPS challenge, but there are sex differences in the mechanisms for this. Neonatal overfeeding also programs some changes in brainstem responses to LPS, while susceptibility to high fat diet remains relatively unaffected. **Summary:** We suggest that avoiding overfeeding in earlier life may help in reducing the risks of HPA axis dysfunction throughout life. We also identify two potential targets for pharmacological treatments to reduce HPA axis inefficiency in the neonatally overfed.

Chapter 1

General Introduction

1.1 Childhood obesity

Overweight and obesity are becoming more and more common in Australia. A record from Australian Bureau of Statistics indicated that 69.9 % of males and 55.2 % of females were classified as overweight in 2014 and 26 % of males and 24 % of females were classified as obese in Australia (ABS 2014). Excess body weight is the cause of heavy burdens on persons, families and society. For instance, the annual direct cost (health care and non-health care) for overweight adults over the age of 30 was approximately $6.5 billion in 2005, whilst the annual direct cost of obesity was $14.5 billion (Colagiuri, Lee et al. 2010). The indirect cost of overweight and obesity was $35.6 billion in this year. The total annual cost in 2005 was $56.6 billion. By 2008, this annual direct and indirect cost was increased to $58.2 billion (Economics 2008). Studies of the latest trends have shown around three quarters of the Australian adult population, including 83% of males and 75% of females aged 20 years and over may be overweight or obese by 2025, thus these costs are likely to continue to increase in the future (Haby, Markwick et al. 2012).

Obese children are a particular concern with respect to these statistics. In 2014, around 24% of boys and 27% of girls in Australia were believed to be overweight or obese (ABS 2014). The percentage of obesity in children has thus increased from one quarter of the population in 2007 (AGDH 2007) to total one third of Australian children in 2013 (ABS 2013). These are likely to continue to the year of 2025 (Haby, Markwick et al. 2012). Obese children are considered significantly more likely to become obese adults (Whitaker, Wright et al. 1997). Non-obese children have approximately a 13% chance of becoming an obese adult (Whitaker, Wright et al. 1997), while an obese child has more than a 50% chance of growing into an obese adult (Whitaker, Wright et al. 1997). Extreme obesity in children is associated with a 70% chance of the obesity progressing into adulthood (Whitaker, Wright et al. 1997).

Studies have not only shown that the obese children are more likely to become obese in adulthood, but they also have a higher risk of developing short and long-term health problem and behaviours, such as type 2 diabetes, cardiovascular disease (AIHW 2004) and excessive responses to stress (Bose, Olivan et al. 2009).

1.2 HPA axis responses to stress in obesity

Stress is any sufficiently intense challenge to homeostasis that harms (physical stress) or threatens to harm (psychological stress) it and results in a generalised non-specific as well as a specific signature response (Schneiderman, Ironson et al. 2005). The body responds to stress by recruiting sympatho-medullary (SAM), hypothalamic-pituitary-adrenal (HPA) axis and behavioural responses. In terms of the HPA axis (Figure 1.1), when we receive stress signals, the paraventricular nucleus of the hypothalamus (PVN) releases corticotrophin-releasing hormone (CRH) into the median eminence (Balbo, Leproult et al. 2010, Lucassen and Cizza 2012). Adrenocorticotropic hormone (ACTH) is an important mediator between the brain and adrenal response to stress. It is released from the pituitary gland upon stimulation by CRH. ACTH then interacts with the adrenal cortex at melanocortin 2 receptor (MC2R) with melanocortin 2 receptor accessory protein (MRAP) as an accessory protein and causes the release of glucocorticoids (GCs) into plasma.

Figure 1.1 Hypothalamic-pituitary-adrenal (HPA) axis

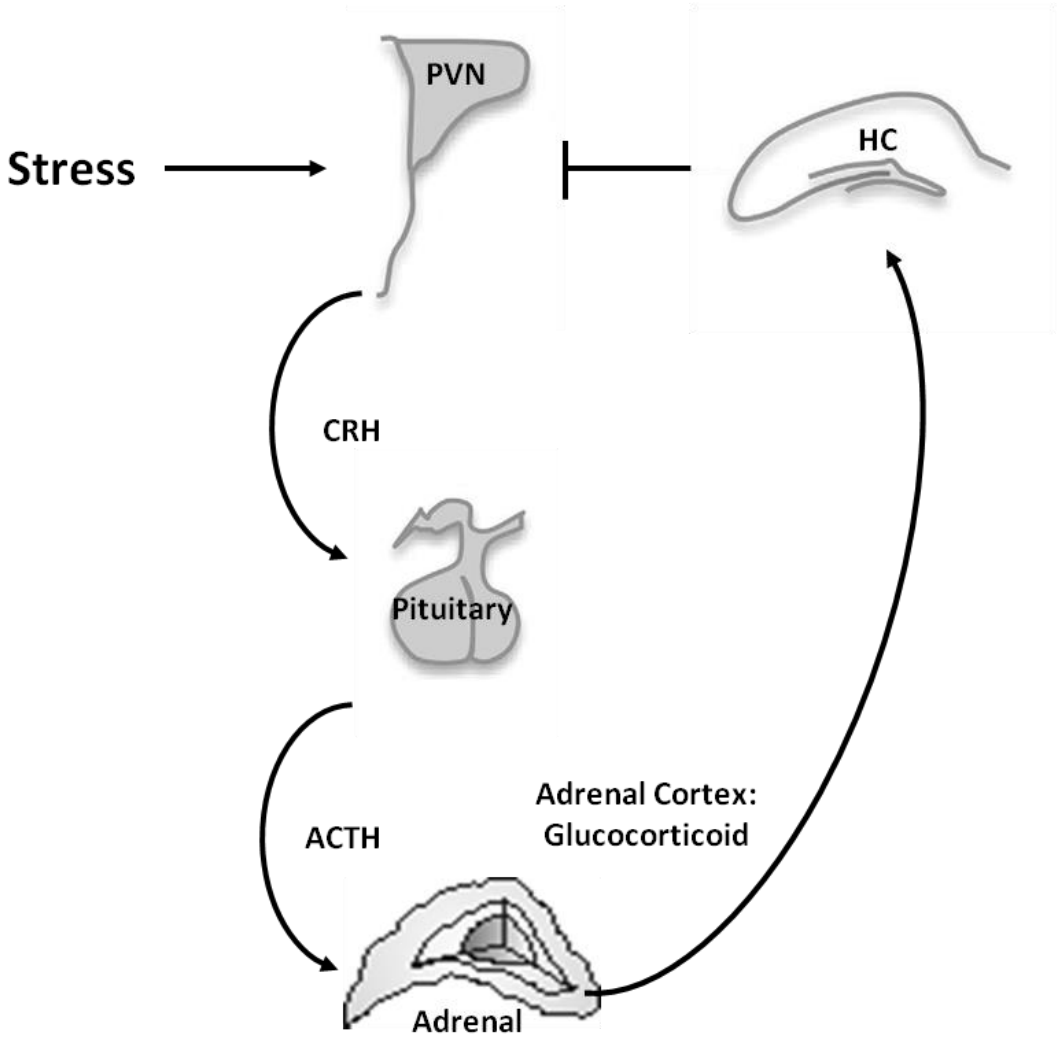


Figure 1.1 Under normal conditions, stressors act at the level of the brain to stimulate hypothalamic-pituitary-adrenal (HPA) axis activation, the paraventricular nucleus (PVN) of the hypothalamus releases corticotrophin-releasing hormone (CRH) into the median eminence stimulating pituitary activation and release of adrenocorticotropic hormone (ACTH), and activates the adrenal to release glucocorticoids (GCs). GCs negatively feed back in the hippocampus and hypothalamus, to suppress further HPA axis activation.

As described above, when the HPA axis is activated in response to stress, the activation will increase GCs (cortisol in humans, corticosterone in rats and mice) release from the adrenal glands within minutes (Sapolsky, Romero et al. 2000, Boonstra 2005, Vera, Zenuto et al. 2012). Cortisol and corticosterone can differentially bind intracellular and

membrane-associated receptors (Schmidt, Malisch et al. 2010). Glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) are the GC receptors necessary for GC negative feedback (Herman, Patel et al. 1989, Eberwine 2006). GR is regulated by the physiological and pharmacological activities of GCs (Oakley and Cidlowski 2013). MR can bind with mineralocorticoids (MCs) (such as aldosterone and its precursor: deoxycorticosterone) and glucocorticoids (Fuller and Young 2005). As previously described, GCs can bind and activate the MR; however, GC concentrations in circulation are higher than MCs, thus, GCs can fully occupy the MR; the 11β-HSD2, that convert cortisol to cortisone, can inhibit GCs binding with MR (Rogerson and Fuller 2000). MR has a higher affinity for GC and GR has a lower affinity. Their distribution in rodent brain is also different: MR is found mainly in limbic areas, especially in the hippocampus, and GR is more widely found around all brain regions (Herman, Patel et al. 1989, Zhe, Fang et al. 2008). From de Kloet's studies, we know that when stressed, the MR expression and the MR / GR ratio are indexes for GCs responses. During the stress, MR is activated and increases the excitability of limbic networks – an area for responsibility of emotion and appraisal. Then, the rising GC levels activate GR, resulting in adjustment to the energy consumption of the limbic-cortical circuits that control behavior and memory storage. When the MR / GR ratio is imbalanced, this can cause dysfunction of the HPA axis. This imbalance is an important mechanism for chronic-stress and depression, and can occur with prolonged GCs synthesis (de Kloet 2014).

GCs have a number of roles in combatting the stressor but also feed back directly and indirectly onto the PVN to inhibit further HPA axis activation and GC release (Turnbull and Rivier 1999, Beishuizen and Thijs 2003). GC release has a day and night cycle. In humans, it peaks in the morning, then the GC levels slowly drop down during the day time, but are increased by meals and in deep sleep around 3 am in a normal sleep cycle (Lucassen and Cizza 2012). 5 – 10% of GC in circulation are in an active free form, 80 – 90% of GC is

combined with GC binding globulin and 10 – 15% GC combines with albumin and sex-hormone binding globulin (Lewis, Mopert et al. 2006, Cizza and Rother 2012). When human GC levels are over 500 nmol/L, the GC binding globulin is fully saturated (Putignano, Dubini et al. 2001, Lewis, Mopert et al. 2006). GC levels can be determined in plasma, saliva and urine (Lewis, Mopert et al. 2006, Lucassen and Cizza 2012). Salivary GC levels can increase within 5 minutes after plasma GC increases and the salivary GC level is 30 – 50 % lower than plasma GC (Putignano, Dubini et al. 2001).

Notably, the HPA axis can function differently in obesity. GC levels in obese people have been recognized as different from average levels (Lucassen and Cizza 2012). When GC levels were analysed in plasma every 2 hour for 24 hour, obese adult males (mean BMI, 35.4 kg/m^2) had lower GC levels compared with non-obese males (mean BMI, 26.8 kg/m^2) (Vgontzas, Pejovic et al. 2007). In other studies, 24 hour urine free cortisol levels did not correlate with BMI or waist circumference (Fraser, Ingram et al. 1999), but GC metabolites such as tetrahydrometabolites were positively correlated with BMI (Fraser, Ingram et al. 1999, Sandeep, Andrew et al. 2005, Baudrand, Campino et al. 2011). Thus, obesity is more likely to increase the production and catabolism of GC without necessarily directly changing plasma GC levels (Lucassen and Cizza 2012).

Obesity can also lead to hyperactive stress responses and mood disorders (Doyle, le Grange et al. 2007, Scott, McGee et al. 2008, Benson, Arck et al. 2009, Abiles, Rodriguez-Ruiz et al. 2010). In USA, more than half of high-risk adolescents that were binge eating at least once in a month reported high levels of depression, anxiety, and stress, and impairments in physical health, emotional functioning, and social functioning (Doyle, le Grange et al. 2007). Benson et al.'s study showed in Germany, presentation stress can lead to heart rate and diastolic blood pressure increases that are significantly higher in obese premenopausal

women than the control group 10 minutes following speaking stress; serum interleukin 6 (IL-6) levels both pre- and post- speaking were also higher in the obese than in controls; and the granulocytes, cluster of differentiation 3 (CD3) positive t-cells and high-sensitivity c-reactive protein (hs-CRP) concentrations were significantly higher in obese women after 45 minutes speaking (Benson, Arck et al. 2009). In New Zealand obese people were significantly more likely to have mood disorders, major depressive disorder, and anxiety disorder (Scott, McGee et al. 2008). Obese patients in Spain had higher levels of stress, anxiety, depression, food craving, and eating behaviour disorder symptoms and lower levels of self-esteem and quality of life compared with normal-weight controls. Patients with type III and type IV obesity differed only in anxiety and personality findings (Abiles, Rodriguez-Ruiz et al. 2010). Thus, obese people are more likely to have to face anxiety, depression and other stress-related disorders than their non-obese counterparts.

The way the HPA axis responds to stress can also be influenced by the early life environment. For instance, maternal nursing can influence rat offspring responses to stress in terms of neuroendocrine factors that program the HPA axis, for example by increasing hippocampal glucocorticoid receptor (GR) expression (Meaney and Aitken 1985, Weaver, Cervoni et al. 2004). Excess grooming and nursing during the first 10 days of life can reduce offspring plasma ACTH and GC levels in adulthood, in response to an acute stress (Liu, Diorio et al. 1997). In addition, over-nursing can reduce the rat offspring curiosity to explore novelty, and significantly decrease CRH receptor density in the locus coeruleus (Caldji, Tannenbaum et al. 1998). Interestingly, these effects can influence females into the second generation (Francis, Diorio et al. 1999).

One early life environmental factor that can have particular long-term effects on HPA axis function is early life nutrition. For human babies, bottle feeding is more likely to

induce overfeeding than feeding from the breast (Li, Magadia et al. 2012), because infants may be encouraged to finish the bottle (Thompson, Mendez et al. 2009). In animal models, raising the rats in small litters has been used for many years to study the immediate and long-term effects of overfeeding during the early postnatal period (Knittle and Hirsch 1968, Miller and Parsonage 1972, Oscai and McGarr 1978). Neonatal overfeeding by reducing litter sizes can program post-weaning obesity in rat pups (Schmidt, Fritz et al. 2001, Morris, Velkoska et al. 2005, Rodel, Prager et al. 2008). Previously our group's studies have shown that rats made obese due to being suckled in small litters, where they had free access to their mother's milk, had HPA axis maturation that was accelerated compared with normally fed controls (Bulfin, Clarke et al. 2011, Spencer and Tilbrook 2011, Cai, Ziko et al. 2016). Particularly, these effects showed sex-dependence, the effects on female rats were more pronounced than males. From the studies of our laboratory, we used immunohistochemistry for Fos as a marker of neuronal activation after restraint stress, and we saw increased activation of PVN neurons in female rats that were raised in small litters compared to controls, but no differences in males. Both males and females that were neonatally overfed in this study manifested an overweight phenotype in adulthood (Spencer and Tilbrook 2009). Interestingly, even though the female rats also had increased body weight gain compared with controls if they were suckled in small litters, they did not display indications of being more anxious than the control groups, even demonstrating reduced anxiety with greater vertical and middle arena exploration, less grooming in the open field, and enhanced open arm exploration in the elevated plus maze (Spencer and Tilbrook 2009).

1.3 The sex differences of HPA axis responses to stress in obesity

The evidence suggests obesity can affect men and women differently in terms of malfunction of HPA axis. Previous studies have shown that giving patients an oral

dexamethasone treatment can stimulate GC negative feedback on the HPA axis in both obese men and women, but only the obese women have blood cortisol and ACTH suppression rates that are significantly higher than in non-obese controls (Pasquali, Ambrosi et al. 2002). Moreover, these sex-dependent differences in the HPA axis can be present from childhood. The study of Jones showed 7 – 9 year old boys' and girls' salivary cortisol responses to stress were dependent upon time of day. In girls, the peak cortisol level in the morning was inversely correlated with birth body weight, whereas in boys, whole day but not morning cortisol levels were inversely correlated with birth body weight (Jones, Godfrey et al. 2006). Furthermore, the HPA axis response to stress has also been shown to be different between genders irrespective of obesity (Verma, Balhara et al. 2011). Men have a higher ACTH response to stress than women, and women have a more sensitive adrenal cortex response to stress than men (Roelfsema, van den Berg et al. 1993).

In our previous studies, neonatal over-nutrition can exacerbate HPA axis responses to psychological stress (restraint) in females long-term, but this does not occur in males; in contrast, neonatal under-nutrition can attenuate HPA axis responses to stress in males long-term, but not females (Spencer and Tilbrook 2009). Overall, obesity can lead to the HPA axis responding differently in males and females and these differences may be programmed from early life, but the mechanisms behind these sex differences remain unclear.

1.4 SAM axis and brainstem catecholamine neuron responses to stress in obesity

In addition to the HPA axis, the SAM axis (Figure 1.2) also plays an important role in the response to stress (Jayasinghe, Lambert et al. 2016). When the animal experiences stress, the adrenal glands will release GCs into plasma as described above. At the same time,

the SAM axis is activated, and stimulates the adrenal glands to secrete catecholamines: adrenaline and noradrenaline, to activate the cardiovascular system to coordinate the response to the changes in homeostasis (Dalin, Magnusson et al. 1993, Jayasinghe, Lambert et al. 2016). Comparatively, the SAM axis acts very acutely (seconds to minutes), and the HPA axis is effective over the medium term (minutes) (Herman and Cullinan 1997, Smith and Vale 2006, Gotlib, Joormann et al. 2008, Gaete 2016). A major brain SAM response to stress is to activate catecholamingeric neurons that project to the PVN to stimulate PVN's activation (Smith and Vale 2006, Bienkowski and Rinaman 2008).

Figure 1.2 Sympatho-adrenal-medullary (SAM) axis

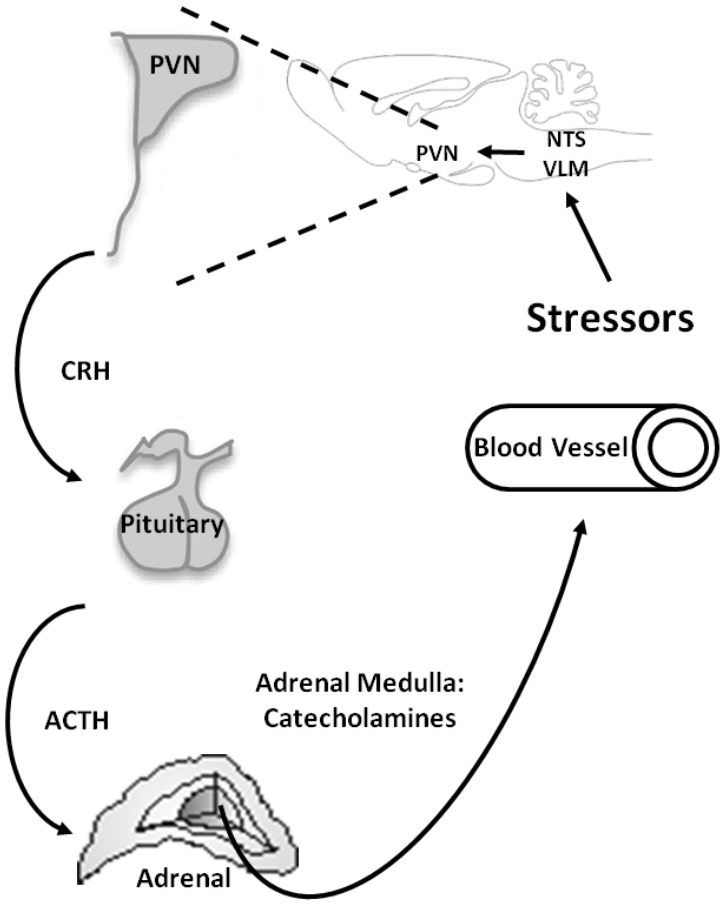


Figure 1.2 The nucleus of the solitary tract (NTS) and ventral medulla (VLM) mediate stress responses to the PVN though catecholamine and non-catecholamine neurons. PVN: paraventricular nucleus of hypothalamus; CRH: corticotrophin releasing hormone; ACTH: adrenocorticotropic hormone.

The PVN directly projects and receives projection from the rat ventrolateral medulla (VLM) and to the nucleus tractus solitarius (NTS) (Shafton, Ryan et al. 1998, Affleck, Coote et al. 2012). The VLM is an important brainstem region involved in control of the activities of the sympathetic nervous system (Dampney 1994, Dampney 1994, Guyenet and Stornetta 2004, Guyenet 2006, Mueller 2010). In addition, injected superoxide dismutase mimetics, tempol and tiron, in rostral VLM and reduced the effects of emotional stress in rabbits. Thus superoxide is a key mediator of excitatory actions of angiotensin II in rostral VLM during acute stress (Mayorov, Head et al. 2004). Sympathetic nervous system activity and oxidative stress in the rostral VLM was significantly higher in obesity-prone rats than in obesity-resistant rats (Konno, Hirooka et al. 2012).

The NTS and VLM are transit regions for immune signal transfer. They receive and integrate incoming immune signals and transfer these signals to forebrain regions to integrate the host defense responses (Elmquist and Saper 1996, Ericsson, Arias et al. 1997, Marvel, Chen et al. 2004, Gaykema, Chen et al. 2007). Moreover, the PVN receives signals that projected from the lateral parabrachial nucleus (PBL), and the PBL receives signals is from the NTS (Herbert, Moga et al. 1990, Elmquist and Saper 1996). Thus, both direct and indirect projections from the brainstem may influence functioning of forebrain regions that mediate sickness responses. NTS and VLM are also activated in response to immune challenges, such as live bacteria (Gaykema, Goehler et al. 2004, Goehler, Gaykema et al. 2005), lipopolysaccharide (LPS) (Wan, Janz et al. 1993, Elmquist, Scammell et al. 1996), or pro-inflammatory cytokines such as interleukin-1β (IL-1β) induced by other pathogens and their related metabolites (Ericsson, Kovacs et al. 1994, Buller, Xu et al. 2001). The above studies have shown all these regions have strong c-Fos-like immunoreactivity to indicate neuronal activation in response to stress and immune challenge (Gaykema, Chen et al. 2007).

The GR expressed in NTS modulates the responses of stress on the endocrine system. If the rats' GR in NTS is antagonised with mifepristone, the endocrine and behavioural stress responses are decreased, plasma corticosterone levels are increased, and open arm exploration in the elevated plus maze is decreased, indicative of increased anxiety-like behaviour (Ghosal, Bundzikova-Osacka et al. 2014). NTS is therefore a key regulator of feeding behaviour. Several clinical and animal studies have shown that the cellular fuel adenosine monophosphate-activated protein kinase is regulated by gastrointestinal and adipose signals at the level of the NTS (Minokoshi, Alquier et al. 2004, Xue and Kahn 2006, Grill and Hayes 2009, Blasi 2016).

Studies from Day and colleagues showed both NTS and VLM catecholamine cells are important, but differential, contributors to the HPA axis responses to stress. This group used systemic hypoxia to stress rats. They found that most of the neurons activated were on NTS noradrenergic cells rather than adrenergic cells (Buller, Smith et al. 1999). A VLM ibotenic acid injection significantly reduced the hypotensive hemorrhage-induced activation of hypothalamic vasopressin, oxytocin and medial PVN cells (Buller, Smith et al. 1999). These findings indicate the NTS and VLM have different roles in the SAM axis responses to physical stress. Moreover, VLM and NTS lesions with ibotenic acid injection also can reduce the IL-1β induced medial PVN CRH cell activation. NTS lesions suppressed the recruitment of central amygdala neurons after stress but this is not shown by VLM lesions (Buller, Xu et al. 2001). Furthermore, physical and psychological stressors: haemorrhage, immune challenge, noise, restraint and forced swim can all induce activation of noradrenergic and adrenergic cells on NTS and VLM with different patterns of activation depending on the stress. Haemorrhage and immune challenge induced Fos activation of NTS and VLM noradrenergic cells that were significantly more rostral than those induced by noise, restraint or forced swim. (Dayas, Buller et al. 2001). Rats with ibotenic acid lesions in VLM have

significantly suppressed CRH cell responses to restraint, and also suppressed neuronal responses of the medial amygdala in the PVN to emotional stress (Dayas, Buller et al. 2001). The PVN can adjust NTS and VLM responses to a lipopolysaccharide (LPS) challenge, and this may result from descending projections to NTS and VLM from the medial and lateral PVN (Buller, Dayas et al. 2003). Thus, the brainstem catecholamine cells of the NTS and VLM are transferring the systemic immune signals to PVN to connect the HPA axis and SAM axis together in response to stress.

Neonatal overfeeding can permanently change HPA axis function, and the SAM axis and HPA axis responses to stress are synergistic. Overfeeding during pregnancy can influence the NTS in female offspring in the mouse. Neuropeptide Y activates NTS in adult mice that were overfed *in utero* so that activation is significantly higher than the control dams' offspring (Wang, Ji et al. 2015). Perenboom et al.'s study that exciting the neonatal rat's airway can significantly increase pro-inflammatory cytokine concentrations in the NTS within a short time (Perenboom, Beckers et al. 1996). Challenging adult male rats with LPS i.p. injection can significantly increase numbers of c-Fos positive cells in the NTS, as well as plasma cortisol levels (Reyes, Abarzua et al. 2012). Overall, the full impact of neonatal overfeeding on the acute central pro-inflammatory response of VLM and NTS to immune challenge is not clear but we hypothesise the catecholamine cell of VLM and NTS response to stress in brainstem will be exacerbated by neonatal overfeeding.

1.5 The relationship between HPA and Sam axes

Medullary catecholaminergic neurons in the NTS and VLM play important roles in the induction of HPA axis responses to immune-related stimuli. Indeed, as described above, LPS or cytokines can cause noradrenaline release in the hypothalamus (Lavicky and Dunn 1995, Pacak, Palkovits et al. 1995, Smagin, Swiergiel et al. 1996). The catecholamine

neurons of the NTS and VLM respond to immune challenges and signal to the PVN (Ericsson, Kovacs et al. 1994). These also suggest the catecholaminergic and non-catecholaminergic pathways can independently mediate the different responses induced by different stressors. Furthermore, the catecholamine cells of the VLM and NTS are activated by circulation of IL-1 signals, which relate a prostaglandin-dependent signal to affect the HPA axis responses in the rat (Buller, Xu et al. 1998), as well as increasing CRH release with the activation of neurosecretory neurons (Ek, Arias et al. 2000). In animal studies, LPS challenged rats showed a large number of tyrosine hydroxylase (TH) cells of the VLM and NTS were positive for c-Fos, and indomethacin (an inhibitor of PG synthesis) largely prevented the VLM, but not the NTS, activation (Lacroix and Rivest 1997). These data indicate that prostaglandins mediate the effects of the acute-phase responses depending on the systemic stressful situation, brain region, cell type, and the activated target genes (Lacroix and Rivest 1997). Thus, the mechanisms of catecholaminergic and non-catecholaminergic contribution to the forebrain processing of the immune signals, and the central immune responses (such as HPA activation) in neonatally overfed animals still need to be discovered.

1.6 LPS as an activator of the HPA and Sam axes

While there are many types of stress that can influence an animal, these can loosely be grouped into psychological stressors, ones that threaten to harm the animal and physical stressors, and those that actually cause harm (Dayas, Buller et al. 2001). Examples of physical stress include haemorrhage, hypoxia, and immune challenge. For the purposes of the studies in this thesis we will be addressing the effects of HPA and axes activation with LPS.

LPS is major component of the outer membrane of Gram-negative bacteria, and leads to a strong response of the immune system in normal animals (Lu, Yeh et al. 2008, Brandenburg, Schromm et al. 2010, Wang and Quinn 2010). Pathogen-associated molecular

patterns of LPS can be recognised by toll-like receptor (TLR)4 (Poltorak, He et al. 1998, Erridge 2010). Then, TLR4 is activated and through the myeloid differentiation primary response gene 88 (MyD88) -dependent and -independent pathway activates nuclear factor κB (NFκB) (Kawai, Adachi et al. 1999). Synthesis of inflammatory mediators such as pro-inflammatory cytokines [e.g. tumour necrosis factor (TNF)α and IL-1β] are initiated via phosphorylation of inhibitory factor κB (IκB) and subsequent translocation of NFκB to the immune cell nucleus, where it activates cytokine transcription (Sawchenko, Imaki et al. 1993). These pro-inflammatory cytokines will stimulate the production of prostaglandins via cyclooxygenase-2 (COX-2), then these prostaglandins act at the ventromedial preoptic area to stimulate heat conservation and production, finally causing fever (Morrison, Nakamura et al. 2008).

From our group, previous and recent studies showed the LPS can increase neonatally overfed adult rats' core temperatures up to 0.5 ^{o}C above those of controls (Clarke, Stefanidis et al. 2012). We also have previously published studies that showed a neuroimmune LPS challenge in early postnatal life can permanently modify the response of an animal's TLR4 expression and HPA axis function (Boisse, Mouihate et al. 2004, Spencer, Martin et al. 2006, Mouihate, Galic et al. 2010), indicating the early developmental period may be particularly vulnerable to challenges to immune function. In this regard, we have seen the secreted proinflammatory cytokines such as TNFα, and IL-1β from immune cells are significantly higher in obese animals after an adult LPS challenge in neonatally overfed rats compared with saline-treated controls (Clarke, Stefanidis et al. 2012). In particular, this increase in circulating proinflammatory cytokine production is associated with reduced IκB phosphorylation in the liver and spleen, which is likely to lead to attenuated pro-inflammatory transcription factors such as NFκB release, then reduced translocation, and less initiation of cytokine transcription, also resulting in reduced activation of prostaglandin E2

(PGE2) to catalyse COX-2 in the brain (Spencer, Galic et al. 2011). At the same time, LPS also increases pro-opiomelanocortin (POMC), the parent peptide for ACTH, presumably leading to more GC release. The HPA axis is thus also playing a significant role in the regulation of responses to immune challenge. Can neonatal overfeeding therefore also lead to the changes in the global and central functions of HPA axis in response to LPS?

Previous work has also shown responses to LPS can differ depending on body weight. From a study in diet-induced obese rats, these have an extended fever response time, increased plasma TNFα, IL-6, and IL-1 receptor antagonist concentrations 8 hours after LPS. White adipose tissue IL-1 receptor antagonist gene expression was also higher in obese than in lean rats after LPS (Pohl, Woodside et al. 2009). These data appear to indicate obese individuals may have a higher chance of facing problems with infections. This idea is supported in the literature, thus, from Buckman's study, we can see that obese rats have macrophage recruitment and inflammation in fat tissues as well as infiltrating monocytes in the central nervous system that are directly correlated with body weight. Moreover, the fat mass and markers of inflammation in fat tissue in particular point out obesity is likely to stimulate the recruitment of immune cells to the central nervous system (Buckman, Hasty et al. 2014). These data suggest that neonatally overfed animals may be more susceptible to LPS if they are also consuming a high fat diet as adults. We will test this in Chapter 4.

As described above, inflammation induced by LPS enhances central and global responses to HPA and SAM axis through an increase in release of GCs and catecholamines to regulate pro-inflammatory cytokines. NTS and VLM have strong c-Fos-like immunoreactivity that is induced by LPS, thus this will be used to indicate neuronal activation. These tests will be shown in Chapter 5.

1.7 The animal model of neonatal overfeeding

In clinical studies, mothers exposed to over- or under-nutrition environments during the gestational period leads to their children having increased risk of becoming overweight adults (Ravelli, Stein et al. 1976, Kensara, Wootton et al. 2005, Muhlhausler, Adam et al. 2006, Taylor and Poston 2007, Wrotniak, Shults et al. 2008, Spencer and Tilbrook 2009). There are similar results in several and animal studies (Taylor and Poston 2007), including in rat pups. The increased weight gain in neonatal overfeeding due to the reduced litter sizes is likely to be due to reduced competition for food among the pups, as well as due to the dam producing milk that is higher in fat (Schmidt, Fritz et al. 2001, Morris, Velkoska et al. 2005, Rodel, Prager et al. 2008, Spencer and Tilbrook 2009). As such, rat body mass indices and fat mass indicate rats from small litters are overweight / obese compared with controls (Figure 1.3) (Clarke, Stefanidis et al. 2012, Stefanidis and Spencer 2012). Thus, over-nutrition during lactation can have effects throughout the animal's life, and lead to obesity in adulthood (Boullu-Ciocca, Dutour et al. 2005, Morris, Velkoska et al. 2005, Spencer and Tilbrook 2009). Whether this accelerated weight gain and accumulation of body mass can be termed "obesity" in a human sense is debatable, but they certainly display some of the complications of obesity and the metabolic syndrome, including stress hyper-responsiveness and changes to immune function. As described above, Wistar rats' litter sizes were manipulated so that pups were suckled in a small litter of four to create a neonatal overfeeding model or 12 for control. The pups from small litters have greater access to their dam's milk, consume milk that is higher in fat, and show accelerated growth and weight gain that is continued into adulthood (Fiorotto, Burrin et al. 1991, Mozes, Sefcikova et al. 2004, Clarke, Stefanidis et al. 2012). Thus, we will simulate an over-nutrition environment during lactation to create the neonatal overfeeding animal model for this thesis.

Figure 1.3 Adult rats BMI and DEXA images

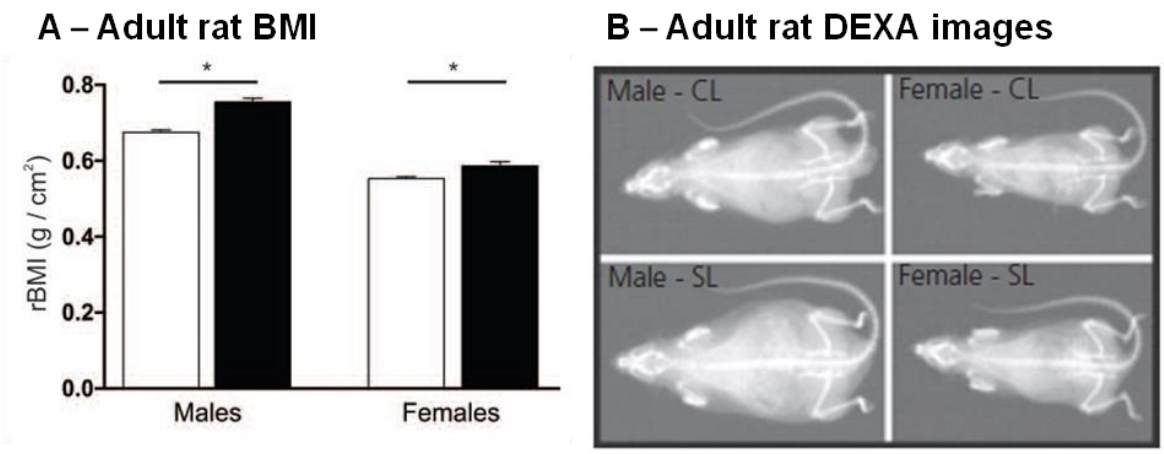


Figure 1.3 Adult rats BMI (Stefanidis and Spencer 2012) and DEXA images (Clarke, Stefanidis et al. 2012). A) Adult rat (P63) BMI. B) Adult (P77) percentage fat as determined by DEXA. n = 8 rats per group. * $p < 0.05$. Data are mean + SEM. A is adapted from: Stefanidis and Spencer 2012. B is adapted from: Clarke, Stefanidis et al. 2012.

1.8 Hypothesis and aim

Over all, we hypothesise that neonatal over-nutrition can influence the function of the HPA axis long-term. In order to test this hypothesis, we have 4 main aims: 1) To determine how the HPA axis is altered in male rats by neonatal overfeeding. 2) To determine how the HPA axis is altered in female rats by neonatal overfeeding. 3) To determine if neonatal overfeeding makes an animal more susceptible to the effects of a high fat diet. 4) To determine how the NTS and VLM in the SAM axis are altered by neonatal overfeeding.

Chapter 2

Neonatal Overfeeding Exacerbates Hypothalamic–pituitary–adrenal axis Responses to Immune Challenge in Male Rats

These data have been published "Guohui Cai, Ilvana Ziko, Joanne Barwood, Alita Soch, Luba Sominsky, Juan C. Molero & Sarah J. Spencer (2016). Overfeeding during a critical postnatal period exacerbates hypothalamic-pituitary-adrenal axis responses to immune challenge: a role for adrenal melanocortin 2 receptors. *Sci Rep*. 6. doi:10.1038/srep21097." A copy of the paper is included in Appendix 1.

2.1 Introduction

As described in Chapter 1, obesity and hypothalamic-pituitary-adrenal (HPA) axis function are interrelated, with obesity exacerbating stress responses and vice versa (Wallerius, Rosmond et al. 2003). Obese individuals tend to have greater stress responses to stressful circumstances and are more likely to change their behaviour in response to stress (Torres and Nowson 2007). Further study has indicated that obesity can change adrenocorticotropic hormone (ACTH) secretion from the pituitary and there are sex differences in this response (Pasquali, Ambrosi et al. 2002). Conversely, stress can also alter feeding and metabolism. For instance, during stress, the fasting insulin, blood glucose, and total-, low density- (LDL), high density- (HDL) lipoprotein cholesterol, synchronize with the release of cortisol (Rosmond, Dallman et al. 1998). If the stress is ongoing, these changes can persist and contribute to obesity. In addition to an important interaction between obesity and the HPA axis in adult life, the early life environment can also make an individual vulnerable to obesity and disrupted stress responses (Ravelli, Stein et al. 1976, Spencer 2012).

The prenatal and postnatal periods can independently program the HPA axis and body weight. In terms of the prenatal environment, parental stress and nutrition levels during gestation can affect the offspring long-term. Babies born to mothers who were pregnant during the Dutch famine of 1944 – 45 were more likely to be obese as adults and have differences in central neural control of food intake, growth, and adipose accumulation (Ravelli, Stein et al. 1976). In adults who went through the famine as babies *in utero*, glucose and insulin levels 120 minutes after a glucose challenge are higher (de Rooij, Painter et al. 2006). Triacylglycerol levels are also significantly higher (de Rooij, Painter et al. 2007). Further tests by de Rooij's laboratory, showed the malnutrition and parental stress of the Dutch famine had a small effect on personality traits and stress appraisal. They also showed

sex- dependence (these findings will be expanded on in Chapter 3.) (de Rooij, Veenendaal et al. 2012). Not only is fat accumulation in adults affected by *in utero* famine, but also the LDL / HDL cholesterol ratios are significantly higher; HDL-cholesterol and apolipoprotein A tended to be lower; total cholesterol, LDL-cholesterol, and apolipoprotein B concentrations in plasma tended to be higher than in control adult (Roseboom, van der Meulen et al. 2000). These factors are strongly linked to cardiovascular disease (Roseboom, van der Meulen et al. 2000).

Animal experiments have shown the exposure to different nutritional environments in the prenatal period can permanently change HPA axis function. Food restricted to half of average in rats during the last week of gestation disturbs the HPA axis development, and reduces the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in hippocampus, levels of corticotropin releasing hormone (CRH) in the hypothalamus, and the concentration of plasma ACTH. The concentrations of corticosterone in newborns are also increased at birth and decreased 2 hour after birth in pups born to food-restricted dams (Lesage, Blondeau et al. 2001). Similarly, *in utero* high fat diet can increase animal HPA axis responses to stress in the offspring (Lesage, Blondeau et al. 2001, Lingas and Matthews 2001, Bloomfield, Oliver et al. 2003).

From postnatal studies it is clear the postnatal lifestyle can also influence the likelihood of a baby developing obesity (Monasta, Batty et al. 2010). For instance, exposure to cigarette smoke after birth can significantly increase childhood overweight and obesity (Huang, Lee et al. 2007); breastfeeding can significantly reduce the risk of childhood obesity (Arenz, Ruckerl et al. 2004). Ong and colleagues have demonstrated that a baby's body weight in early postnatal life can significantly predict their adult body weight, with overweight babies being more likely to be obese adults (Whitaker, Wright et al. 1997, Ong

2006, Oddy 2012). Postnatal lifestyle can also influence HPA axis function long-term (Bose, Olivan et al. 2009, Maniam, Antoniadis et al. 2014). In order to study the effect of and mechanisms for the influence of early over-nutrition through life, we use a model of litter size manipulation so that rat pups are suckled in small litters (where they have access to more milk) or control litters. Our recent research has shown that neonatal overfeeding can permanently change the adult rodents' HPA axis (Bulfin, Clarke et al. 2011, Clarke, Stefanidis et al. 2012). We also show differences in neonatal nutrition can change the rats' behavioural responses in adulthood, with differences between male and female rats. Male rats that are made smaller by being suckled in a large litter have reduced anxiety-like behaviour in the elevated plus maze (Bulfin, Clarke et al. 2011). Also, these smaller males have less activation of the paraventricular nucleus of the hypothalamus (PVN) in response to the psychological stress, restraint.

Previous studies from our group have also investigated the changes in the febrile and neuroimmune responses of juvenile and adult, male and female, Wistar rats made obese by overfeeding during the postnatal period. Our group found that the febrile responses to an immune challenge with bacterial mimetic lipopolysaccharide (LPS) are exacerbated in these rats (Clarke, Stefanidis et al. 2012). The neonatally overfed rats' core temperatures were higher after LPS compared to controls. This finding is associated with enhanced pro-inflammatory cytokine release into circulation (Clarke, Stefanidis et al. 2012). Plasma pro-inflammatory cytokine interleukin (IL)-6, tumor necrosis factor (TNF)α, and IL-1β concentrations are nearly three times higher in neonatally overfed rats after LPS than in controls. HPA axis activation is also exacerbated. The neonatally overfed rats have about twice as many neurons activated in the PVN, as measured by immunohistochemistry for c-Fos, and a slower corticosterone response (Bulfin, Clarke et al. 2011). We also see the neonatally overfed rats have an increased expression of toll-like receptors (TLR)2 and 4 in

adipose tissue and higher phosphorylation of inhibitory factor κB (IκB) (Clarke, Stefanidis et al. 2012). Adipose TLR3 has similar changes, but the response to a viral mimetic which acts at TLR3 is not different (Clarke, Stefanidis et al. 2012). This work illustrates that the early life diet can influence HPA axis in the long-term, potentially interacting with feeding and metabolic factors to increase body weight gain, but the mechanisms for these changes were unknown. Therefore, in the present study, we examined how postnatal over-feeding alters indices of HPA axis function in adult rats and what steps of the HPA axis were altered.

In order to examine how neonatal overfeeding alters the HPA axis, we time-mated Wistar rats as described in our previous studies (Clarke, Stefanidis et al. 2012). We manipulated litters into "small" (4 pups) and "control" (12 pups), and used cross-fostering to reallocate the newborn rats to avoid the original parent. After postnatal day (P) 21 the pups were paired with the same sex and fed a normal rodent chow diet. We then performed the experiments when the rats were grown to adulthood, approximately postnatal day 70, considered similar in age to adult age of humans (Quinn 2005). When the rats reached adulthood, we examined several aspects of the HPA axis including GR and MR gene expression to test for the potential for glucocorticoid (GC) negative feedback to be altered. We also investigated changes in other hormones, genes, and proteins involved in the stress axis in adulthood, under basal conditions and in response to an immune challenge that stimulates the HPA axis, LPS. Here we show the central HPA axis response to LPS in the neonatally overfed rats tends to be normal but that neonatal overfeeding suppresses and slows the LPS-induced melanocortin 2 receptor (MC2R)-mediated GC release from the adrenal gland; thus, suppressing GC negative feedback on the HPA axis.

2.2 Methods and materials

2.2.1 Animals

Timed pregnant Wistar rats were purchased from the Animal Resources Centre (Animal Resources Centre, Murdoch, WA, Australia). These rats were maintained on a 12:12 hour light cycle (the lights were on at 7:00 am), and kept at 22 +/- 1 ^{o}C, and on pelleted rat chow diet (nutritional information: 19.4% protein, 4.8% fat, 4.8% fibre, 14.0 MJ/kg energy; Specialty Feeds Pty Ltd, WA, Australia), and tap water. The resources were available *ad libitum*. On the day of the pups birth, all pups were removed from their biological dams and were randomly allocated to new dams, avoiding original dams. The litter sizes of pups were set at either 4 (small litter, overfed, SL) or 12 (control litter, normal, CL) pups, with an equal ratio of males and females. Excess pups were culled. The pups' body weights were recorded at P0, P7, P14, and P21 as whole litters until weaning. On the day of weaning (P21), the pups were separated by sex and housed as littermate pairs. Normal animal husbandry was supplied twice a week. The rats were maintained under the same conditions as the dams and allowed to grow to adulthood (approximately P70). All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals. All these were approved by the RMIT University Animal Ethics Committee (see Appendix 3).

2.2.2 Intraperitoneal LPS

On the day of experimentation (P70 +/- 1 week), the rats were given intraperitoneal (i.p.) injections of either LPS (100 µg/kg in 1 mL/kg pyrogen-free saline *Escherichia coli* serotype 026:B6; L-3755; Sigma, St Louis, MO, USA) as we have previously described (Tapia-Gonzalez, Garcia-Segura et al. 2011, Clarke, Stefanidis et al. 2012, Ye, Huang et al.

2012, Ziko, De Luca et al. 2014), or an equivalent volume of pyrogen-free saline. Hyperion capillary tubes were used to collect the blood samples from tail nicks immediately before (0 minute) and 15, 30, 60, 90, 120 minutes after LPS injection. The plasma samples were obtained from blood samples after centrifugeation at 15000 rpm for 10 minutes at 4 ^{o}C. The plasma samples were stored at -80 ^{o}C until further use.

2.2.3 Tissue collection

The rats were deeply anaesthetised with Lethabarb (approximately 150 mg/kg pentobarbitone sodium, i.p.) 120 minutes after the LPS injection. A group of rats was used for flash-frozen brain tissue collection, used in gene analysis, and a group of rats was used for brain fixation and immunohistochemical analysis. From the first cohort, the hippocampus, hypothalamus, pituitary and adrenal samples were quickly dissected, snap frozen in liquid nitrogen, and stored at -80 ^{o}C until further use. Cardiac perfusion was used to obtain fixed brain. The brain samples were perfused transcardially with phosphate buffered saline (PBS) at 4 ^{o}C, at pH 7.4 followed by 4% paraformaldehyde (PFA) in PBS at 4 ^{o}C, at pH 7.4, with a flow rate of 50 mL/min. Then the brains were removed and post-fixed for 4 hours in the same fixative before being transferred to cryoprotectant with 20% sucrose in PBS at 4 ^{o}C. The forebrain samples were cut into 30 µm coronal sections using a cryostat. All experiments were initiated between 9:00 am and 12:00 pm to limit potential effects of circadian rhythms on any parameters measured.

2.2.4 Fos immunohistochemistry

Neuronal activation was assessed in the fixed brains by quantifying Fos-positive-immunoreactivity. Briefly, a one-in-four series of forebrain sections from each animal was incubated in primary rabbit polyclonal Fos antibody (Santa Cruz Biotechnology, Santa Cruz,

CA, USA) overnight at 4 ^{o}C, at 1:10,000. Then the forebrain sections were incubated in secondary biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) 1:200, for 2 hours, at room temperature. We then transfered the forebrain sections into an avidin-biotin horseradish peroxidase (HRP) complex A and B (Vector Elite kit, Vector) for 1 hour, at room temperature. At this point, the forebrain sections were incubated in nickel diaminobenzidine for 10 minutes at room temperature to visualise the HRP activity. Then, added hydrogen peroxide (Sigma-Aldrich Australia Pty Ltd, Australia) into diaminobenzidine solution (0.0015% H_2O_2) to incubate tissue continually, until staining developed (seen as a black nuclear deposit). The reaction was terminated once there was an optimal contrast between specific cellular and non-specific background labelling. The coloured forebrain sections from each treatment group were processed simultaneously, and mounted on poly-L-lysine-coated slides, dehydrated in a series of alcohols, cleared in histoclear and coverslipped.

2.2.5 Cell counts

An experimenter blinded to the group treatments performed cell counts of Fos-positive nuclei in PVN. Cell counts were made at two sections per animal (~1.8 and 1.96 mm caudal to bregma).

2.2.6 Corticosterone assay

Corticosterone concentrations in plasma were measured using a standard corticosterone ELISA kit (Abnova Corp., Taipei, Taiwan). The inter-assay variability for this assay was 7.2% CV, the intra-assay variability was 4.8% CV, and the lower limit of detection was 40 pg⁄mL. All plasma samples were analysed together in duplicate.

2.2.7 rt-PCR analysis

To determine whether neonatal overfeeding affected the HPA axis, we examined several genes relevant to HPA axis function. Ribonucleic acid (RNA) was isolated from hippocampus, hypothalamus, pituitary and adrenal tissue using QIAzol and an RNeasy purification kit (QIAGEN Pty Ltd, Victoria, Australia). RNA (1 µg) was transcribed to complementary deoxyribonucleic acid (cDNA) using an iScript cDNA synthesis kit (Bio-Rad Laboratories Pty., Ltd., Gladesville, New South Wales, Australia), according to the manufacturer's instructions. rt-PCR was performed using Taqman Gene Expression Assays (Applied Biosystems, Mulgrave, Victoria, Australia). A Bio-Rad iQ5 was used to assay the signal. Fold differences in target messenger RNA (mRNA) expression were measured using the delta-cycle threshold method by comparison with the housekeeping gene, 18S (Livak and Schmittgen 2001, Schmittgen and Livak 2008), and expressed as mRNA relative fold change e.g. (Galic, Riazi et al. 2009, Mouihate, Galic et al. 2010).

Table 2.1 TaqMan probe details (Life Technologies) used for rt-PCR

Target Gene	NCBI Reference Sequence	TaqMan Assay ID	Amplicon Length
Nr3c1	NM_012576.2	Rn00561369_m1	73
Nr3c2	NM_013131.1	Rn00565562_m1	79
Crh	NM_031019.1	Rn01462137_m1	112
Avp	NM_016992.2	Rn00690189_g1	78
Pomc	NM_139326.2	Rn00595020_m1	92
Mc2r	NM_001100491.1	Rn02082290_s1	126
Mrap	NM_001135834.1	Rn01477212_m1	62
Tlr4	NM_019178.1	Rn00569848_m1	127
18s	X03205.1	4319413E	187

2.2.8 Corticosterone responses to adrenocorticotropic hormone

To determine to what extent changes in the HPA axis were due to changes in the adrenal gland itself, we measured the corticosterone responses to stimulation of the adrenal gland with ACTH (1.5 µg/kg in 1mL/kg pyrogen-free saline, s.c. injection, single dose). CL and SL adult rats were given ACTH and we took blood samples via tail nick immediately prior to injection and 0, 15, 30, 60, and 90 minutes after injection. Samples were analysed as described above.

2.2.9 Western blot analysis

As we saw differences in the expression of the MC2R gene in the adrenal gland between the groups, we attempted to assess if protein levels of MC2R were also altered by neonatal overfeeding, using Western blot. Proteins (10 - 20 µg) were separated by 10% sodium dodecyl sulphate polyacrylamide gel using a Bio-Rad mini gel mould system and Bio-Rad PowerPac™ HC for electrophoresis. Proteins were transferred to Bio-Rad Immun-Blot® Low Fluorescence PVDF Membrane. Membranes were incubated overnight at 4 ^{o}C with primary rabbit polyclonal MC2R antibody (Santa Cruz Biotechnology, USA), at 1:1000, then the membrane was transfered to secondary biotinylated anti-rabbit antibody in 1:3000 at room temperature for 2 hours. Protein bands were detected after application of enhanced chemiluminescence substrate (ECL) (Thermo Fisher Scientific Australia Pty Ltd, Scoresby Victoria, Australia) using a Bio-Rad ChemiDox to develop the signal. The membrane was subsequently stripped with β-mercaptoethanol (Sigma-Aldrich Australia Pty Ltd, Australia). Then, membranes were re-used to detect total β-actin as the housekeeping protein. Briefly, the membrane was incubated in primary rabbit polyclonal β-actin antibody (Santa Cruz Biotechnology), in 1:1000 overnight, at 4 ^{o}C, and then incubated in secondary biotinylated anti-rabbit antibody in 1:5000 dilution at room temperature for 2 hours. Protein bands were

detected after application of ECL. The results were calculated as the ratio between MC2R and β-actin. Unfortunately, the antibody quality was insufficient for us to reliably determine relative MC2R protein levels. We also attempted primary antibody concentrations of 1:200 and 1:500, secondary antibody concentrations of 1:2000 and 1:5000 without success. We show examples of these blots in the "Supplementary Data" section of this thesis. Others have reported that there is currently no adequate antibody available for this protein (Park, Walker et al. 2013).

2.2.10 Data analysis

IBM SPSS 22 was used for statistical analyses and GraphPad was used for preparation of graphs. We analysed males and females separately. Results from the males are reported here. Results from the females are reported in Chapter 3. To compare pre-weaning body weights between CL and SL rats, an analysis of variance (ANOVA) with repeated measures was used, with litter size as the between factor and age as the repeated measure. When a significant interaction was found between litter size and age, Student's unpaired t-tests were performed for each time point. Since a significant effect of age on weight was expected and not the primary subject of our investigation, we made an *a priori* decision to limit our individual comparisons to the effect of litter size. Thus, once a significant interaction between age and litter size was found, the appropriate comparisons were between the two litter size groups at each age, so only t-tests are necessary. Adult parameters were compared using multi-factorial ANOVAs with litter size, adult diet, and LPS treatment as between factors where appropriate, with Tukey's *post hoc* comparisons where significant main effects or interactions were found. We also included time (minutes) as a repeated measure in analysis of plasma corticosterone concentrations. Data are presented as the mean + standard error of the mean (SEM). Statistical significance was assumed when $p < 0.05$.

2.3 Results

2.3.1 Neonatal overfeeding leads to increased rat body weight throughout life

As we have shown previously (Clarke, Stefanidis et al. 2012), there was a significant interaction between litter size and age on pre-weaning body weights (Figure 2.1A). The SL rats were significantly heavier than the CL rats at P14 and P21. There were no differences on the day of birth or at 7 days. The body weights of the adult SL male rats were still significantly heavier than their normally fed brothers on the day of tissue collection (Figure 2.1B).

Figure 2.1 Pre-weaning total and male adult body weights

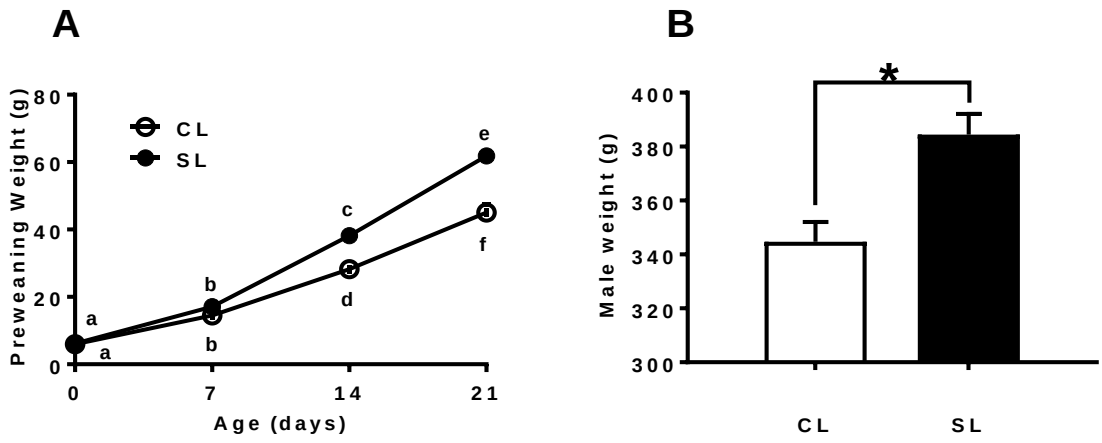


Figure 2.1 A) Pre-weaning total body weights. Pups were weighed in whole litter units, neonatal overfeeding (SL) is associated with accelerated growth during the suckling period compared with control (CL) $F_{(3,66)}$ = 30.46, $p < 0.001$, and every 7 days until weaning day. Note: standard error bars are included but are too small to be detectable for this data set. B) Male adult body weights. This weight phenotype was maintained into adulthood $F_{(1,90)}$ = 23.30, $p < 0.001$. Different letters indicate differences between the groups. "a, b, c, d, e, and f" compared with different groups, $p < 0.05$, n = 8 litters per group, CL: 12 pups per litter, SL: 4 pups per litter. * Compared with CL group, $p < 0.05$, n = 8 animals per group. All data are mean ± SEM.

2.3.2 Neonatal overfeeding exacerbates the HPA axis response to LPS

Our group has previously shown that male rats suckled in the SL group have more activated neurons in the PVN after a single dose of LPS in adulthood, compared with the CL group, as assessed by numbers of Fos-immunoreactive cells (Bulfin, Clarke et al. 2011, Clarke, Stefanidis et al. 2012). We replicate this finding in the present experiments. In the present study, the results showed that the single dose of LPS significantly activated the PVN region in SL rats, but not CL (Figure 2.2A). The LPS injection also caused a dramatic plasma corticosterone increase in the CL group with a peak at 60 minutes, and corticosterone levels that returned towards baseline by 90 or 120 minutes. In SL rats, the plasma corticosterone responded to the LPS injection more slowly, and was still higher at 120 minutes after LPS injection. (Figure 2.2B).

Figure 2.2 Male: PVN neuronal activation and plasma corticosterone levels in responses to LPS

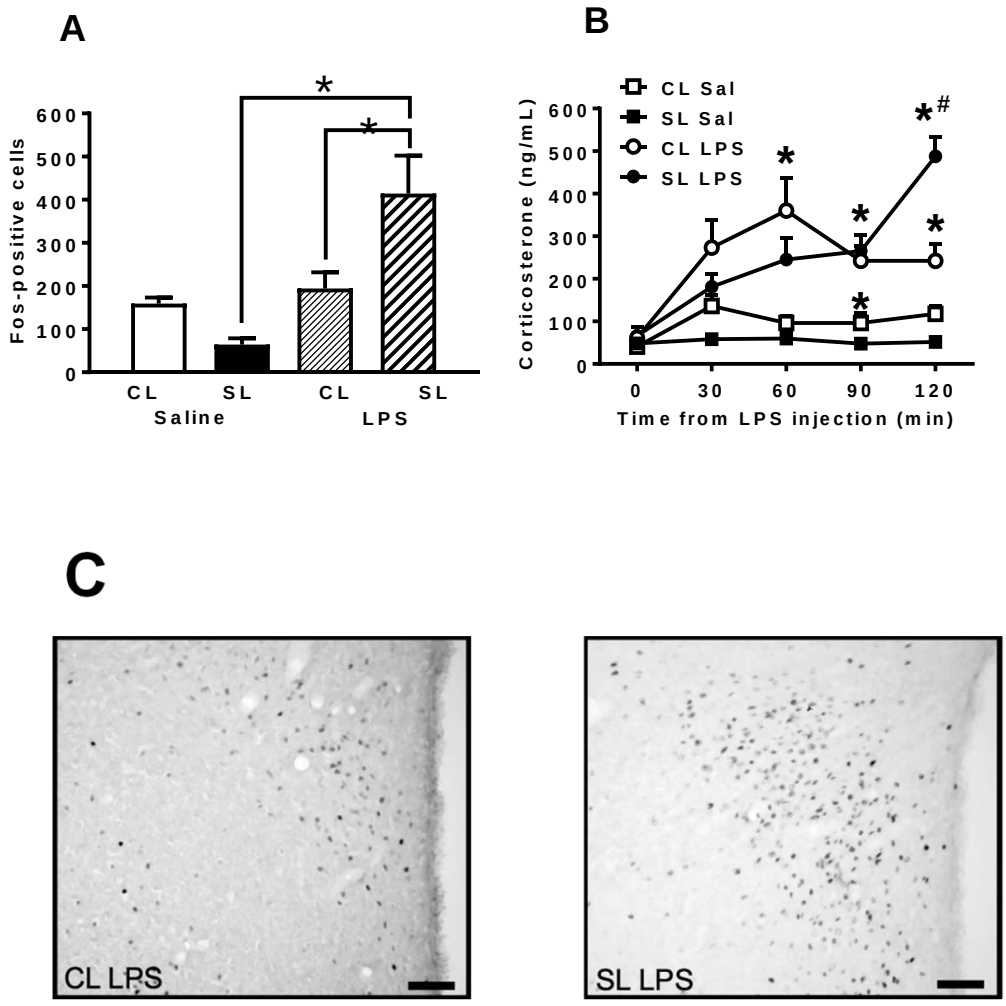


Figure 2.2 A) Paraventricular nucleus of the hypothalamus (PVN) neuronal activation (Fos) in response to LPS; significant litter size x LPS interaction $F_{(1,19)} = 9.46$, $p = 0.006$; *: Compared with CL group, CL Saline: n = 6, SL Saline: n = 5, CL LPS: n = 6, SL LPS: n = 7 animals per group. B) Plasma corticosterone in response to i.p. LPS; significant effect of litter size × LPS × time interaction $F_{(4,128)} = 7.46$, $p < 0.001$; *: Compared with saline-treated group for the same litter size, #: Compared with CL-LPS, $p < 0.05$, n = 8 animals per group. All data are mean + SEM. C) Photomicrographs of the PVN from representative CL and SL LPS-treated rats. Scale = 100 µ m.

2.3.3 The GR and MR responses to LPS in the hypothalamus and the hippocampus after neonatal overfeeding

Neonatal overfeeding intensified the male rats' responses after the LPS injection, which led us to investigate several aspects of the HPA axis that may be responsible for these changes. We first examined hypothalamic and hippocampal expression of GR and MR, under basal conditions and 2 hours after LPS treatment to assess if the capacity for GC negative feedback was altered in SL rats. In this experiment, GR gene expression in the hippocampus was significantly increased 2 hours after LPS treatment in SL rats but not in CL rats (Figure 2.3A). There were no significant differences in the GR and MR gene expression in the hypothalamus between the male SL and CL rats (Figure 2.3D and E).

Figure 2.3 Male: GR and MR gene expression in hippocampus and hypothalamus

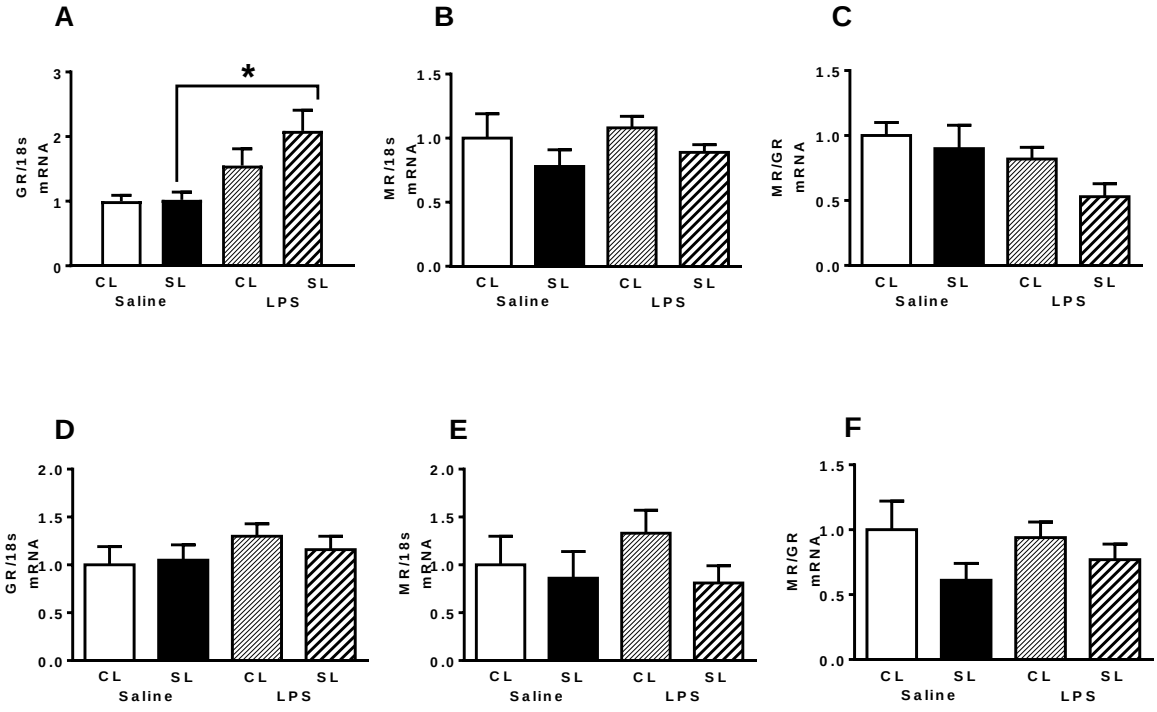


Figure 2.3 A – C) Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) gene expression in hippocampus. D – F) GR and MR gene expression in hypothalamus. A) Significant effect of LPS treatment $F_{(1,26)} = 10.11$, $p = 0.004$; * $p < 0.05$, n = 8 animals per group. All data are mean + SEM.

2.3.4 Neonatal overfeeding does not alter the CRH and AVP responses to LPS in the hypothalamus, and does not alter the POMC responses to LPS in the pituitary gland

Since our analysis of the GR and MR expression suggested GC negative feedback is unlikely to be altered by neonatal overfeeding, we next analysed potential changes in various genes in the hypothalamus and pituitary which are regulated by HPA axis function. In the hypothalamus, CRH is upstream of ACTH and facilitates its release from the anterior pituitary (Balbo, Leproult et al. 2010, Lucassen and Cizza 2012). In present experiments, the SL rats had a reduced expression of CRH after LPS, while expression was unaffected in the CL rats (Figure 2.4A). Hypothalamic arginine vasopressin (AVP) is a hormone involved in water retention and blood vessel constriction (Marieb and Hoehn 2016). It also interacts with CRH to facilitate ACTH release. Our analysis revealed AVP gene expression levels were not significantly different between the groups (Figure 2.4B). At the pituitary, pro-opiomelanocortin (POMC) is the precursor for ACTH and is indicative of the potential for ACTH release into plasma (Rousseau, Kauser et al. 2007). We found that there was a significant main effect of the litter size on pituitary POMC expression, but no significant differences between the SL and CL rats after the LPS challenge with *post hoc* analysis (Figure 2.4C).

Figure 2.4 Male: Gene expression in HPA axis

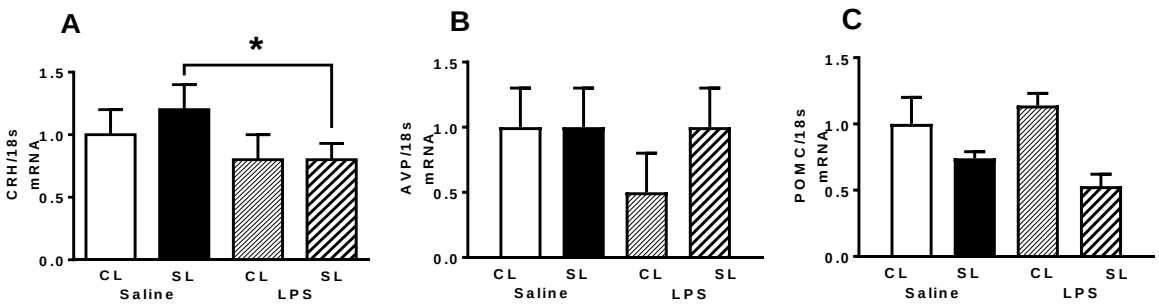


Figure 2.4 A) Hypothalamic gene expression of corticotropin-releasing hormone (CRH); significant effect of LPS $F_{(1,29)} = 7.34$, $p = 0.012$; n = 8 animals per group, B) Hypothalamic gene expression of arginine vasopressin (AVP), CL Saline: n = 7, CL Saline: n = 6, SL LPS: n = 7, SL LPS: n = 8 animals per group. C) Pituitary gland gene expression of pro-opiomelanocortin (POMC). * $p < 0.05$, n = 8 animals per group. All data are mean + SEM.

2.3.5 Neonatal overfeeding suppress the MC2R responses to LPS in the adrenal glands

The downstream link between ACTH and GC release is the MC2R. As described in the introduction (Chapter 1), when the HPA axis is stimulated, ACTH is released from the anterior pituitary and then acts at the MC2R on the adrenal cortex to regulate GC production and release. Therefore, we examined expression of the MC2R gene and that of its accessory protein, MRAP. Our experiment revealed there were no significant effects of the neonatal overfeeding on rats' absolute adrenal weights or the adrenal weights as a percentage of their body weight (Figure 2.5A and B). 2 hour after the LPS challenge, adrenal MC2R gene expression was significantly elevated in SL and CL rats (Figure 2.5C). In SL rats, the MC2R gene expression was significantly lower than in CL rats (Figure 2.5C). There were no significant differences in MRAP gene expression (Figure 2.5D) between the SL and CL rats with *post hoc* analysis, but there was a significant main effect of litter size. There were no

significant differences in MC2R gene expression between the SL and CL rats when we tested expression at 30 minute or 24 hour after LPS injection (Figure 2.5E), but expression of MC2R was suppressed overall at 24 hour relative to saline injection, as was MRAP (Figure 2.5F). Since we found significant differences in gene expression of MC2R, we attempted to assess the protein levels of MC2R in adrenal glands. In the present study, we were not able to detect the protein due to non-specific bands with the commercial antibodies that could not be eliminated. These data are shown in "Supplementary Data" section.

Figure 2.5 Male: Adrenal weights and adrenal / body weights percentage, MC2R and MRAP gene expression in adrenals

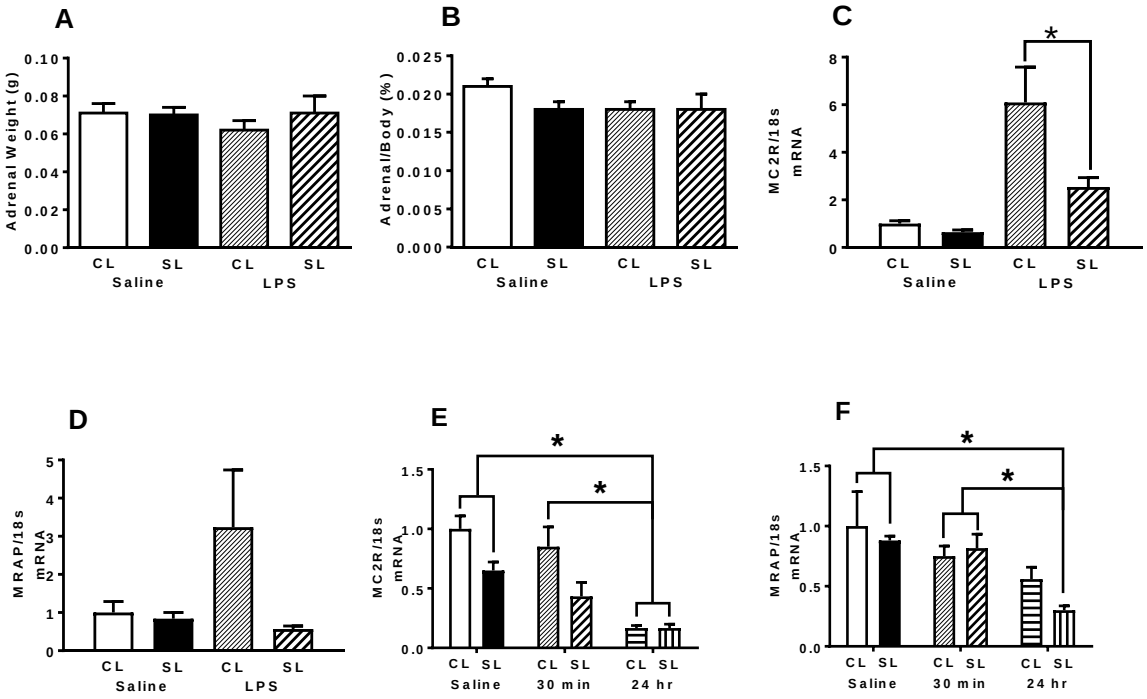


Figure 2.5 A) Absolute adrenal weight. B) Adrenal weight as a percentage of total body weight C) Adrenal gland gene expression of melanocortin 2 receptor (MC2R) under basal conditions and 2 hour after LPS injection; effect of litter size x LPS interaction $F_{(1,26)} = 8.94$, $p = 0.006$; *: $p < 0.001$, CL Saline: n = 7, SL Saline: n = 8, CL LPS: n = 8, SL LPS: n = 8 animals per group. D) Adrenal gene expression of melanocortin receptor accessory protein (MRAP) under basal conditions and 2 hour after LPS injection. E) MC2R gene expression which were on 30 minute or 24 hour after LPS; *: $p < 0.001$, n = 6 animals per group. F) MRAP gene expression 30 minute or 24 hour after LPS; *: $p < 0.05$, n = 6 animals per group. All data are mean + SEM.

2.3.6 Neonatal overfeeding stimulates corticosterone release in the adrenal gland in response to ACTH

The above experiment revealed the neonatally overfed rats were likely to have significant differences in the ability of the adrenal cortex to respond to the ACTH generated by LPS treatment. We therefore measured the corticosterone response to an ACTH challenge in SL and CL rats. In the present results, we found the corticosterone response to ACTH challenge was small and was not significantly different from the response to saline in CL rats. In SL rats, the corticosterone was significantly increased relative to saline at 30 minute after ACTH injection (Figure 2.6A), and was indicative of a robust adrenal response in these rats.

2.3.7 Neonatal overfeeding does not alter TLR4 gene expression in adrenal gland

The experiments presented above suggested the effect of neonatal overfeeding on MC2R and GC production after LPS treatment was likely to be mediated by LPS acting directly at the adrenal glands. For these reasons, we therefore examined if expression of TLR4 (LPS receptor) was altered in adrenal glands. There were no significant differences between SL and CL rats in TLR4 gene expression to indicate LPS would act differently at this site (Figure 2.6B).

Figure 2.6 Male: Plasma corticosterone in response to ACTH,

adrenal TLR4 gene expression

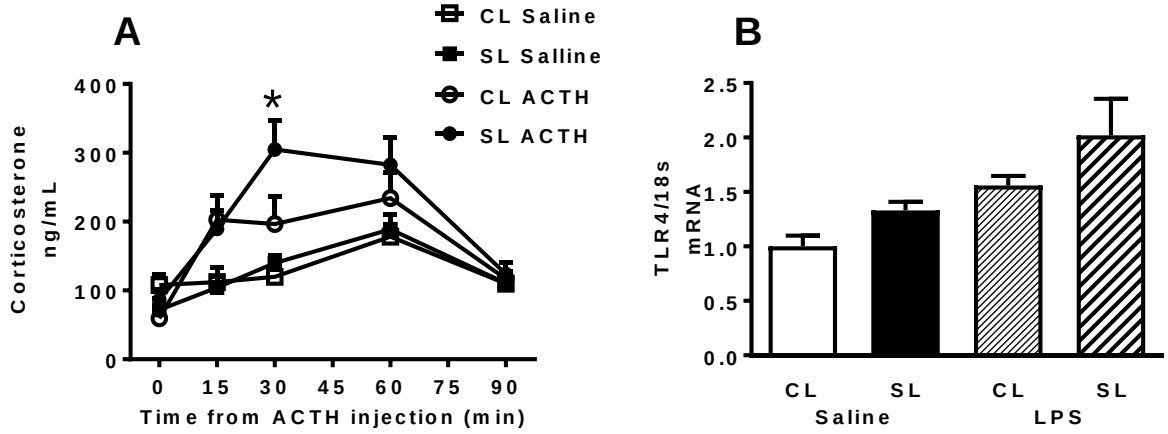


Figure 2.6 A) Adrenocorticotropic hormone (ACTH)-induced plasma corticosterone release in 90 minute following injection. Significant time × ACTH interaction, $F_{(4,128)} = 7.93$, $p < 0.001$; time × ACTH interaction; $F_{(4,128)} = 2.31$, $p < 0.061$; n = 9 animals per group B) TLR4 gene expression in adrenal glands 2 hour after LPS. *: Compared with saline-treated group for the same litter size, $p < 0.001$ CL/SL Saline and CL LPS: n = 7 animals per group, SL LPS: n = 6 animals per group. All data are mean + SEM.

2.4 Discussion

Results from this study support previous findings from our group that show the HPA axis is modified in neonatally overfed male rats compared with controls. Previous studies from our group showed the neonatally overfed male rats had more neurons activated in the PVN (as measured by immunohistochemistry for c-Fos) than controls (Clarke, Stefanidis et al. 2012). In the present experiments, there was a similar increase in c-Fos positive cells in neonatally overfed male rats after LPS compared with controls that confirmed the neonatally overfed male rats have an exacerbated HPA axis response to immune challenge. Another index of HPA axis function is plasma GC secretion. The normal male rats' plasma corticosterone was increased at 30 minutes after LPS i.p. injection, then dropped down back to normal at the 60 and 90 minute time points (Clarke, Stefanidis et al. 2012). The neonatally overfed rats had a corticosterone response that slowly increased and was still elevated at the 90 minute time point. In the present experiment, we extended the time points to 120 minutes and compared the LPS treated male rats with those given a 0.9% saline i.p. injection. Saline-challenged male rats' corticosterone in plasma was stable at each time point. The neonatally overfed male rats' corticosterone slowly increased after 90 minutes to the 120 minute time point. The control rats also responded similarly to previous studies with corticosterone that increased fast and reached a peak at 60 minute, and returned back to baseline at the 90 minute and 2 hour time point.

As the neonatally overfed rats' GC response to LPS was slower and the PVN response exacerbated we hypothesised the GC negative feedback at the level of the hypothalamus or hippocampus would be affected with our model. Effects on GC negative feedback have been seen previously in other studies with different types of change to the early life environment (Liu, Diorio et al. 1997, Champagne, Francis et al. 2003, Weaver,

Cervoni et al. 2004, Zhang, Labonte et al. 2013). If given low levels of maternal care in early life, rats have suppressed hippocampal GR gene expression. This leads to an exacerbation of the HPA axis responses to stress. However, there are no changes in GR gene expression in our neonatally overfed male rats. Hippocampal GR gene expression was significantly increased in overfed male rats, but not controls after 120 minutes LPS challenge, but the CL and SL group were not different from one another with *post hoc* tests. There was also no significant difference between overfed and control rats under basal conditions i.e. after a 0.9% saline injection. Moreover, the MR expression and the MR / GR ratio, which are important indices for responsiveness to GCs (Joels, Karst et al. 2008, de Kloet 2014), were also not different between the groups. These results showed that the neonatal overfeeding was not influencing the HPA axis response to LPS by altering negative feedback at the hypothalamus and hippocampus. In addition, the CRH and AVP gene expression showed no difference between the groups after LPS injection, indicating that the ability of the PVN CRH cells to respond to LPS is probably intact in neonatally overfed. Pituitary POMC gene expression was also not significantly different between the groups, indicating the ability of the pituitary to produce ACTH when the HPA axis was stimulated by LPS was probably also not affected by neonatal overfeeding.

Together, our findings suggest the exacerbated HPA axis responses to LPS in the neonatally overfed are due to changes in adrenal MC2R gene expression. The MC2R gene is significantly and dramatically increased after LPS challenge in male rats fed with control diet during the neonatal period, but in the neonatally overfed, the response to LPS is comparatively suppressed. These MC2R gene expression changes indicate that the overfed male rats may be less able to efficiently respond to the LPS-induced ACTH to stimulate GC release.

Based on the results above, we attempted to analyse the protein levels of MC2R by Western blotting. Previous studies from Lightman's group reported there were no good antibodies for MC2R (Park, Walker et al. 2013) and our trials support this conclusion. We chose the MC2R antibody from Santa Cruz as a trial, as there was an image from rodents with a published reference (Cirillo, Hassona et al. 2012). However, we were not able to detect the protein due to non-specific bands that we could not eliminate. We attempted multiple troubleshooting experiments with this antibody. We always saw strong non-specific bands at around 25 kDa. We also saw weak bands a little below the 33 kDa molecular weight (MW), but no bands at the expected MW, 42 kDa (see Supplementary: Supplementary 1A). The MW of 42 kDa was reported by Uschold-Schmidt (Uschold-Schmidt et al., 2012), due to the two molecular sites for N-linked glycosylation in the extracellular N-terminal region that increases its size. We could not recognise the presence or absence of non-specific bands in the cited publication from the images when using this antibody as these areas of the images were not included. Therefore, the appearance of strong non-specific bands, the missing bands at the expected 42 kDa, the necessity to overexpose the gel to visualise weak bands at the expected MW, meant we could not confirm the blotting was accurate. We also compared the antibodies available from Abcam, Aviva, and Antibodies Online. For all these, the target had similar sequences in the same gene, making them have the same issues with non-specificity and insufficient sensitivity. Moreover, none of these antibodies had online reviews or publications with images for rodent tissue. The MC2R from Abcam has not been tested in rodents. The Aviva one is the same sequence as the Abcam. Finally, we ran the blotting again for the extremely light band around 25 kDa and the results support our rt-PCR data, but we cannot be confident these results represent MC2R real protein levels (Supplementary: Supplementary 1C). There is also the possibility that there are very low levels of MC2R protein in the adrenal.

Based on our results with the rt-PCR, we hypothesised that the GC response to ACTH would also be reduced in neonatal overfeeding rats. Interestingly then, the ACTH actually stimulated more GC release in neonatally overfed than in control rats. In addition to LPS stimulating GC release via the HPA axis, LPS can also directly affect the adrenal glands to release GCs. As such, some studies have shown either ACTH or LPS *in vitro* could stimulate the adrenal glands to produce GCs, aldosterone and expression of steroidogenic enzymes (Huang, Chiang et al. 2010, Liu, Zhu et al. 2011). TLR4 gene expression appears to be high when the ACTH-induced GC release is inhibited (Kanczkowski, Tymoszuk et al. 2011). We therefore hypothesised LPS could act directly on the adrenal glands to affect the MC2R-mediated GC release, and this could be influenced by the neonatal overfeeding. Our previous studies also considered that the neonatal overfeeding might primarily affect the adrenal gland response to LPS, rather than the HPA axis directly as we showed that central and GC responses to restraint stress are normal in neonatally overfed male rats (Spencer and Tilbrook 2009). However, our present study indicated that the TLR4 gene expression in adrenal glands was not significantly different between the groups. In further studies, Dr. Sominsky from our group has extended the finding of the present experiment. This work found the neonatally overfed rats have impaired *in vitro* release and inefficient *in vivo* suppression of ACTH-stimulated corticosterone (Cai, Ziko et al. 2016). We found ACTH stimulated significant adrenal corticosterone release in CL but not SL rats, with a significant difference at 30 minute after exposure to ACTH. These results mean neonatal overfeeding leads to the rats being less able to respond to stimulation of the HPA axis, likely due to their reduced ability to increase expression of the MC2R. In order to study if the differences between the *in vivo* responses to LPS and to ACTH were due to a direct effect of LPS at the adrenal gland, Dr. Sominsky also stimulated adrenal glands *in vitro* with LPS and examined the corticosterone responses. We found LPS mildly suppressed the adrenal corticosterone

release in control rats, with significant differences from baseline at 30 and 45 minute after LPS exposure, but there were no effects in neonatally overfed adrenals (Cai, Ziko et al. 2016).

Recent studies showed that non-genomic intra-adrenal, negative feedback and suppression of the ACTH-mediated GC secretion, is fast, within a few minutes (Walker, Spiga et al. 2015). These might provide a mechanism to explain the differences we found in this study. However, the exact changes behind these molecular mechanisms remain to be discovered. In future studies, it will be useful to examine kinetics and binding efficiency of ACTH on adrenal MC2R in response to stress. Alternatively, there is the possibility that the increased body weight (and possibly increased body fat composition) increased the volume or distribution of corticosterone. This would lead to more corticosterone being sequestered into the tissues, leading to a reduced/slower corticosterone response when measured from the plasma. Additionally, neonatal overfeeding could have altered the concentration of corticosterone binding globulins, leading to a slower increase in free-corticosterone levels in the plasma.

Based on our results, we conclude the central HPA axis response to LPS in the neonatally overfed rats tends to be normal. The sensitivity of the adrenal glands to downstream activation by LPS is reduced. Thus, neonatal overfeeding suppresses the LPS-induced and MC2R-mediated release of GCs from the adrenal glands. This slower GC release leads to less efficient GC negative feedback on the HPA axis and less efficient suppression of the NFκB-mediated transcription of cytokines. At present the molecular mechanisms behind these changes remain to be discovered. However, the neonatally overfed males may have less efficient adrenally-mediated responses to bacterial endotoxin and a reduced ability to respond the bacterial infection (Figure 2.7).

Figure 2.7 HPA axis functions in neonatally overfed male rat

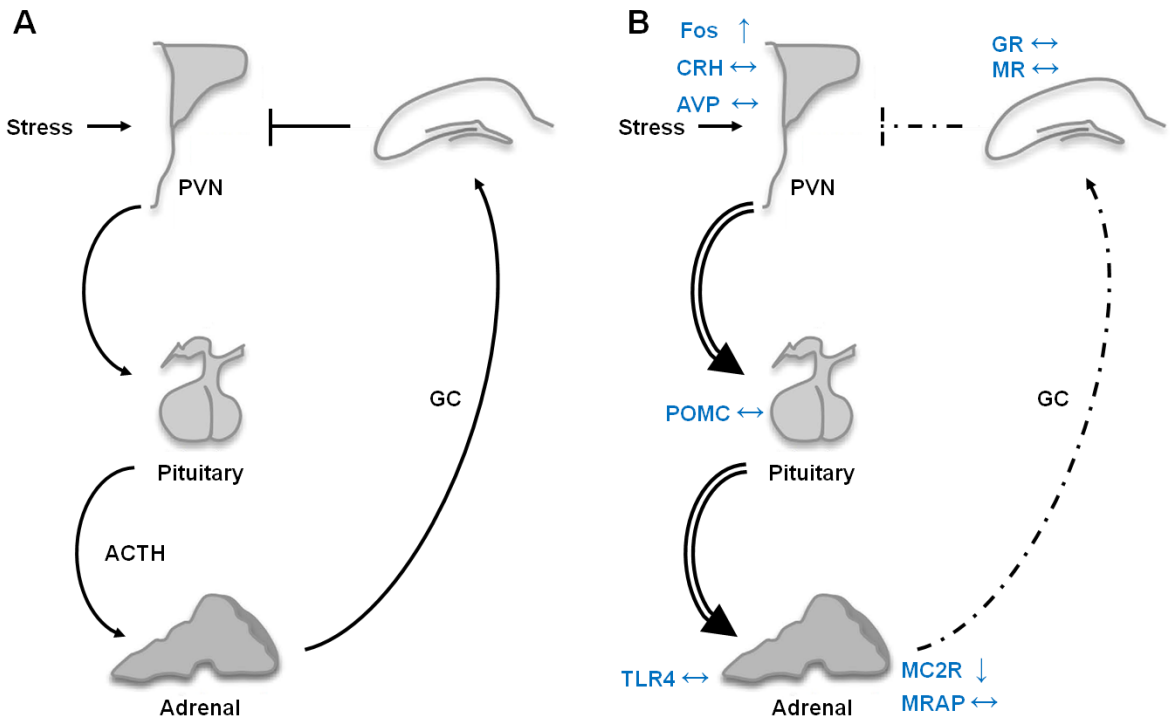


Figure 2.7 A) Under normal conditions: Lipopolysaccharide (LPS) acts at the level of the brain to stimulate hypothalamic-pituitary-adrenal (HPA) axis activation and glucocorticoid (GC) production. GCs feed back centrally to suppress further HPA axis activation. B) In neonatally overfed rats: Central HPA axis responses to LPS are likely to be normal, stimulating paraventricular nucleus of the hypothalamus (PVN) activation and adrenocorticotropic hormone (ACTH) release from the pituitary. The ability of GCs to suppress further HPA axis activity at the level of the brain is also normal. However, the effect of ACTH on the adrenal is impaired leading to slower LPS-induced activation of MC2R-mediated GC release, slower GC negative feedback and exaggerated PVN neuronal activation. Blue arrows indicate direction of gene differences between control and neonatally overfed groups after LPS treatment. AVP, arginine vasopressin; CRH, corticotropin releasing hormone; GR, glucocorticoid receptor; MC2R, melanocortin 2 receptor; MR, mineralocorticoid receptor; MRAP, melanocortin receptor accessory protein; POMC, pro-opiomelanocortin.

Chapter 3

Neonatal Overfeeding Exacerbates Hypothalamic–pituitary–adrenal Axis Responses to Immune Challenge in Female Rats

3.1 Introduction

Work presented in the previous chapter has shown neonatal overfeeding leads to significant changes in male rat hypothalamic-pituitary-adrenal (HPA) axis. We found the numbers of Fos-immunoreactive cells in the paraventricular nucleus of the hypothalamus (PVN) of male neonatally overfed adult rats were significantly higher than in controls after a systemic immune stimulus. It is likely this is at least partially due to the neonatal overfeeding affecting expression of adrenal melanocortin 2 receptor (MC2R) since MC2R gene expression was elevated in control but not neonatally overfed male rats and there was a slower resulting corticosterone response to lipopolysaccharide (LPS). These findings showed neonatal overfeeding not only causes body weight changes that can increase the risk of system-wide disorders (such as cardiovascular disease and diabetes) (Plagemann, Heidrich et al. 1992), it can also influence the HPA axis response to an immune challenge. One caveat to the findings of Chapter 2, however, is that these results are exclusively in male rats. It is still unclear how the nutritional environment in early life influences the function of the HPA axis in females.

In Australia, overweight and obesity affect more adult men than women, the statistics indicate that 56.3% of females were classified as overweight or obese in 2014 – 2015, while in men 70.8% were overweight or obese (ABS 2015). Statistics from children in 2015 shows 26.4% of Australian girls aged 5 to 17 were believed to be overweight or obese compared to 28.5% of boys (ABS 2015). Statistics also predict this trend will continue until to at least 2025 (Haby, Markwick et al. 2012). Although these statistics indicate HPA axis changes in obesity may be more of a problem for men than women, girls will not only have the problems common to boys and men, such as cardiovascular disease and diabetes, but may also develop gynecologic and obstetric complications during adolescence and long-term.

Although the obese and overweight during childhood and adolescence in both girls and boys can lead to impairments in sexual maturation and reproductive dysfunction, girls have to face menstruation dysregulation, dysmenorrhea, high-risk sexual behaviour and inefficient contraception, polycystic ovary syndrome, bone density abnormalities, macromastia, and breast and endometrial cancer (Sukalich, Mingione et al. 2006, Elizondo-Montemayor, Hernandez-Escobar et al. 2016). Furthermore, obese adolescent girls are considered to have more risks associated with pregnancy and parturition, including preeclampsia, gestational hypertension, gestational diabetes mellitus, primary cesarean delivery, and induction of labor (Sukalich, Mingione et al. 2006, Halloran, Marshall et al. 2012, Habbout, Li et al. 2013). In addition, children of obese mothers have more chance of complications, such as pre-term or post-term delivery, macrosomia, meconium aspiration, respiratory distress, stillbirth (Stillbirth Collaborative Research Network Writing 2011, Warshak, Wolfe et al. 2013). These data mean overweight and obese girls may have more chance of facing serious health problems than non-obese girls. Additionally, very early studies have shown obesity can affect childhood self-esteem differently in boys and girls (Kaplan and Wadden 1986). In particular, obese girls are more likely to develop depression than obese boys (Strauss 2000), and this may be affected by many diverse factors, including different cultural backgrounds (Lee, Cheah et al. 2012) and the girls' sensitivity (Elizondo-Montemayor, Hernandez-Escobar et al. 2016). These increases in the likelihood of depression and low self-esteem have been linked with HPA axis dysfunction.

In terms of HPA axis dysfunction, evidence suggests obesity can affect men and women differently. For instance, Pasquali and colleagues used oral dexamethasone to stimulate glucocorticoid (GC) negative feedback on the HPA axis in obese men and women. The results showed obese women have blood cortisol and adrenocorticotropic hormone (ACTH) suppression rates that are significantly higher than controls but obese men do not

(Pasquali, Ambrosi et al. 2002). Moreover, these sex-dependent differences and changes to the HPA axis are evident from childhood. In a study by Jones, 7 – 9 year old boys' and girls' salivary cortisol responses to stress were analysed. Morning peak cortisol levels of girls were inversely correlated with birth body weight. Boys also had cortisol levels inversely correlated with birth body weight, but not in the morning peak period (Jones, Godfrey et al. 2006). Thus, obesity may impact the HPA axis differently in males and females and these differences may be evident from early life.

In our previous animal studies, we found that overfeeding in early life exacerbates HPA axis responses to psychological stress in females long-term, but does not affect males in this regard. Thus, females that were overfed as neonates displayed less anxious behaviour in the elevated plus maze test and had exacerbated PVN neuronal activation in response to 15 minutes restraint stress, while neonatally overfed males were not different from controls (Spencer and Tilbrook 2009). We have also shown neonatal underfeeding attenuates HPA axis responses to stress in males long-term, but does not affect females (Clarke, Stefanidis et al. 2012, Spencer 2012). Together, these findings suggest neonatal nutrition does not affect the HPA axis of males and females in the same way. Therefore, we next wanted to examine the effects of neonatal overfeeding on the female HPA axis.

To test this we continued to use our neonatally overfed rodent model. We examined responses to an immune challenge with LPS at all levels of the HPA axis in females as for males described in Chapter 2.

3.2 Methods and materials

We used female (n = 8 per group) rats generated from the same litters as the males used in Chapter 2 (i.e. their female siblings). All methods and analyses are the same as those used in Chapter 2. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals. All these were approved by the RMIT University Animal Ethics Committee (see Appendix 3).

3.3 Results

3.3.1 Neonatal overfeeding increases female rat body weights throughout life

The female rats for this study were included in the pre-weaning body weights for Chapter 2. As described in Chapter 2, there was a significant interaction between litter size and age on pre-weaning body weights when all the pups (males and females) were weighed as a unit, with neonatally overfed (SL) weighing more than control (CL) at postnatal day (P)7 and P14 (Figure 2.1A of Chapter 2). As we saw for the males, SL female rats were still heavier than the CL female rats into early adulthood (Figure 3.1).

Figure 3.1 Adult female body weights between control and small litter

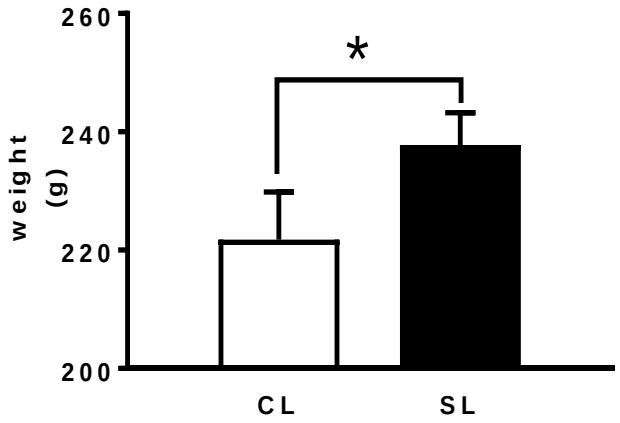


Figure 3.1 Control (CL) and small litter (SL) adult female body weights, * $p < 0.05$, n = 8 animals per group. Data are mean + SEM.

3.3.2 Neonatal overfeeding exacerbates female GC responses to LPS

Some previous results from our group showed SL male rats have greater HPA axis

responses to immune challenge as indicated by greater numbers of Fos-immunoreactive cells in the PVN region after LPS, as well as exacerbated GC release from the adrenal glands after stress in adulthood (Clarke, Stefanidis et al. 2012, Spencer 2012, Cai, Ziko et al. 2016) and we replicated these data in Chapter 2. We have repeated this experiment here in female rats. However, neuronal activation in the PVN in response to the LPS was similar between the CL and SL groups (Figure 3.2A). Analysis of plasma GC levels showed GC levels were significantly elevated with respect to saline in the female CL and SL LPS-treated group at 90 and 120 minutes after LPS treatment, while GC levels in CL did not significantly differ from SL, (Figure 3.2B).

Figure 3.2 Female: PVN neuronal activation and plasma corticosterone levels in responses to LPS

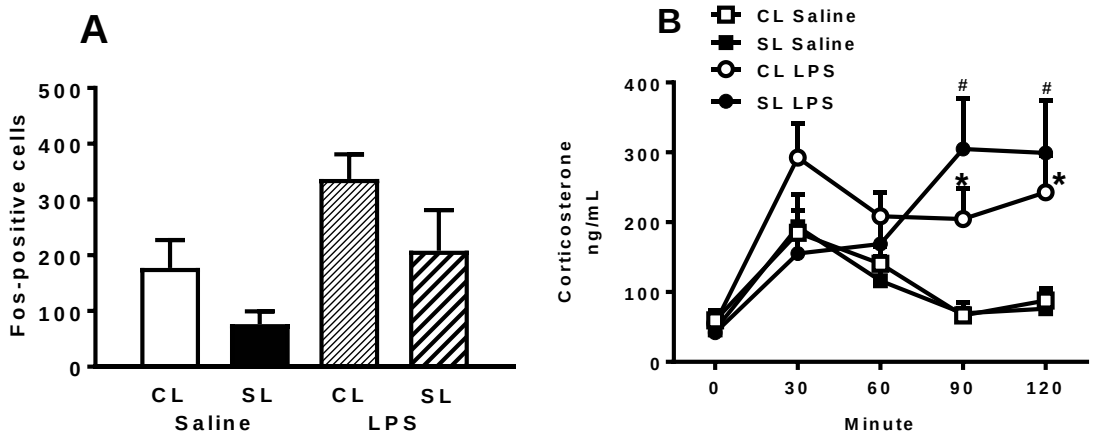


Figure 3.2 A) Paraventricular nucleus of the hypothalamus (PVN) neuronal activation by Fos-positive cells in response to i.p. LPS. B) Plasma corticosterone levels in response to LPS; 90 minute significant of LPS treatment [$F_{(1, 38)} = 19.47$, $p < 0.0001$], 120 minute significant of LPS treatment [$F_{(1, 38)} = 15.3$, $p = 0.0004$]. *: Compared with the saline-treated group, $p < 0.05$; #: Compared with the saline-treated group, $p < 0.05$. n = 7 animals per group. Data are mean + SEM.

We see a seeming separate reaction between the GC concentrations and PVN neuronal activation in that GC were elevated by LPS in the SL group, but Fos-positive cells

in the PVN were not affected differently. These findings may indicate that the neonatally overfed female group may have differences in the sensitivity of the adrenal gland in response to an immune stimulus. We thus investigated if GC responses to ACTH and expression of MC2R and melanocortin 2 receptor accessory protein (MRAP) were affected by neonatal overfeeding the results were shown on Figure 3.3.

3.3.3 Neonatal overfeeding does not change MC2R, MRAP or TLR4 responses to LPS in the adrenal glands of female rats

As described in Chapter 1, when the HPA axis is stimulated by stress, ACTH is released from the anterior pituitary, and then ACTH acts at the MC2R on the adrenal cortex to regulate GC production and release. Therefore, we examined the expression of the MC2R gene and its accessory protein gene MRAP. Our experiment revealed there were no significant litter size differences in the absolute adrenal weights or the adrenal weights as a percentage of total body weight in the female rats (Figure 3.3A and B). When we tested MC2R expression at 30 minutes after LPS injection, there were no significant differences between the female SL and CL groups after saline or LPS (Figure 3.3C). 2 hours after LPS challenge, there was a significant main effect of LPS and litter size, but no *post hoc* differences (Figure 3.3D).

MRAP gene expression was also not significantly different between the CL and SL groups in female rats either under basal conditions or at 30 minutes or 2 hour after LPS injection (Figure 3.3F). Since elevated GC release in response to LPS could not be accounted for by an increase in MC2R expression, we tested if there were any gene expression changes to indicate LPS directly acts toll-like receptor (TLR)4 on immune cells and the adrenal gland. Therefore, we asked if the neonatal overfeeding altered TLR4 gene expression after LPS exposure in the female rats. We saw no significant *post hoc* differences between female SL

rats and the female CL rats in adrenal TLR4 expression, but LPS treatment suppressed TLR4 overall (Figure 3.3G).

Figure 3.3 Female: Adrenal weight, adrenal / body weights percentage and MC2R and MRAP gene expression in adrenals

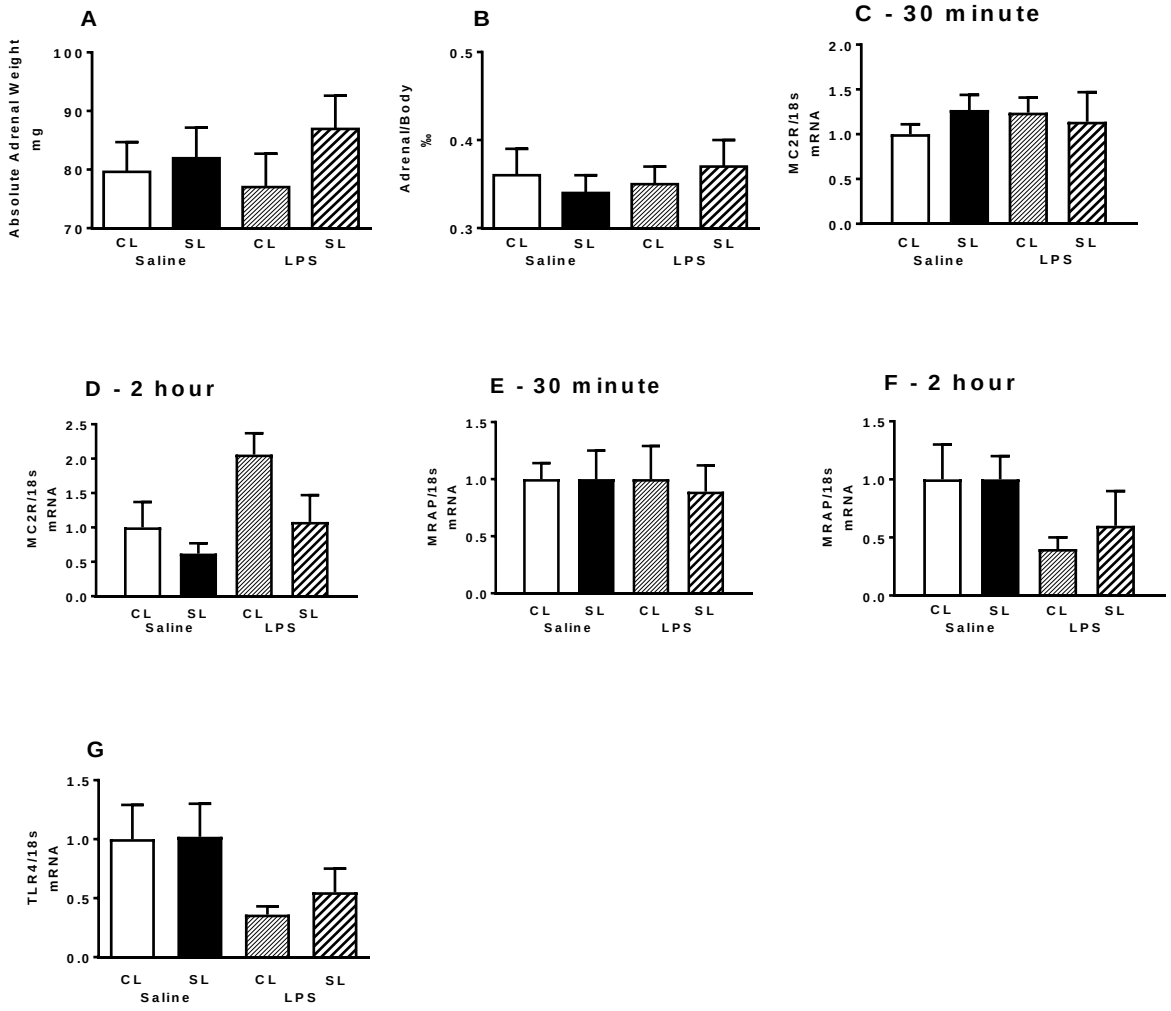


Figure 3.3 A) Absolute female rat adrenal weights. B) Female rats' adrenal weights as a percentage of total body weight. C) Expression of melanocortin 2 receptor (MC2R) gene in adrenal glands 30 minutes after LPS injection. D) Expression of MC2R gene in adrenal gland 2 hours after LPS injection; significant effect of litter size [$F_{(1, 26)} = 5.732$ $p = 0.0242$] and LPS treatment [$F_{(1, 26)} = 4.588$ $p = 0.0417$] but no *post hoc* differences. E) Melanocortin receptor accessory protein (MRAP) gene expression in the adrenal glands 30 minutes after LPS injection. F) MRAP gene expression in adrenal gland 2 hours after LPS injection. G) TLR 4 gene expression in adrenal gland; significant effect of LPS treatment [$F_{(1, 25)} = 5.627$ $p = 0.0257$]. CL Saline and CL LPS: n = 7, SL Saline and SL LPS: n = 8 animals per group. Data are mean + SEM.

3.3.4 Neonatal overfeeding does not alter the CRH and AVP responses to LPS in the hypothalamus of female rats

The elevated GC in response to LPS in SL females did not seem to be due to alterations in the machinery responsible for GC signaling and release at the adrenal. Therefore we hypothesized that corticotrophin-releasing hormone (CRH) and / or arginine vasopressin (AVP) in the PVN might be hypersensitive to LPS, such that the same number of cells was activated in the PVN. We analysed the potential changes in various genes expressed in the hypothalamus and pituitary that are regulated by HPA axis activity. In the present experiments, female CL and SL rats had no differences in CRH gene expression 2 hours after LPS injection (Figure 3.4A). The AVP gene expression was also not affected by neonatal overfeeding or adult LPS (Figure 3.4B).

Figure 3.4 Female: Gene expression in HPA axis

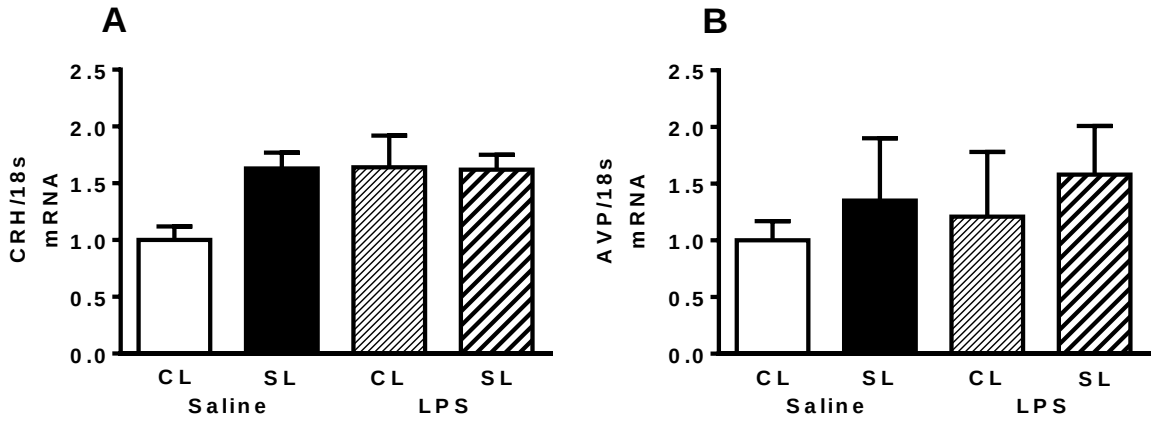


Figure 3.4 A) Expression of corticotropin-releasing hormone (CRH) in hypothalamus. B) Expression of arginine vasopressin (AVP) in hypothalamus. CL Saline and CL LPS: n = 7, SL Saline and SL LPS: n = 8 animals per group. Data are mean + SEM.

3.3.5 Neonatal overfeeding does not alter the POMC responses to LPS in the pituitary gland but leads to hyper-responsiveness to the stress of injection in female rats

We next hypothesised that altered pro-opiomelanocortin (POMC) at the pituitary may be the reason for the slower GC release in neonatally overfed female rats after LPS stimulation. While we saw no differences in POMC expression, indicating the capacity of the pituitary to release ACTH was unaffected by neonatal overfeeding, we did see some differences in the corticosterone produced in response to ACTH (Figure 3.5A).

All groups showed an increase in corticosterone in response to s.c. injection, whether with saline or with ACTH. In the female CL saline-treated rats, corticosterone was approaching baseline and relatively lower than the other groups at 60 and 90 minutes after ACTH injection while SL corticosterone remained high (Figure 3.5B). These results indicated SL female rats were hyper-sensitive to the stress of injection. They also suggested the lack of differences in hypothalamic neuronal activation despite elevated GC release after LPS could be due to impaired GC negative feedback in the hippocampus and hypothalamus since the GC response to ACTH alone was not exacerbated in the SL rats.

Figure 3.5 Female: POMC gene expression in pituitary and plasma corticosterone in response to ACTH

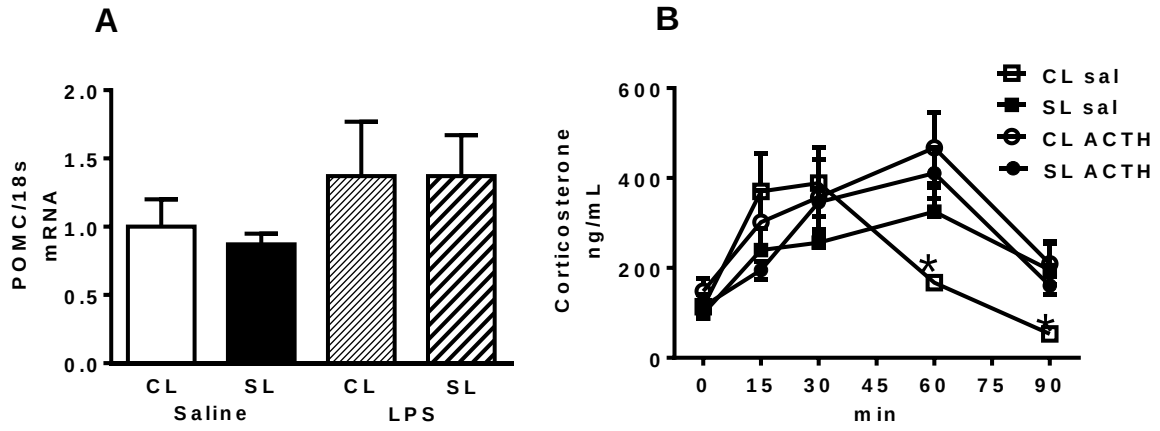


Figure 3.5 A) gene expression of pro-opiomelanocortin (POMC) in pituitary glands. B) adrenocorticotropic hormone (ACTH) -induced plasma corticosterone. Corticosterone: significant effect of interaction [$F_{(12, 160)}$ = 2.446, p = 0.006] and groups [$F_{(4, 160)}$ = 16.82, p < 0.0001). *: compared with SL saline group, p < 0.05, n = 9 animals per group. Data are mean + SEM.

3.3.6 Neonatal overfeeding alters the GR and MR responses to LPS in the female hypothalamus and the hippocampus

We next investigated if GC negative feedback is likely to be less effective in SL females compared with CL. Thus we examined hypothalamic and hippocampus GR and MR expression, under basal conditions, and also 2 hours after LPS injection. In this experiment, LPS injection increased GR gene expression in the hippocampus of female CL rats, but not in the SL groups (Figure 3.6A). Conversely, MR expression was significantly higher in female SL groups after LPS, compared with CL (Figure 3.6B). As such, female CL rats had a lower MR / GR ratio after LPS than the saline and LPS-treated CL groups and the ratio in the female SL groups was unaffected by LPS (Figure 3.6C).

In the hypothalamus, LPS increased GR expression and the ratio between MR and GR in both groups (Figure 3.6D and F), but increased MR expression in the CL group only (Figure 3.6E). Together these data indicate GC negative feedback may be affected by neonatal overfeeding.

Figure 3.6 Female: GR and MR gene expression in hippocampus and hypothalamus

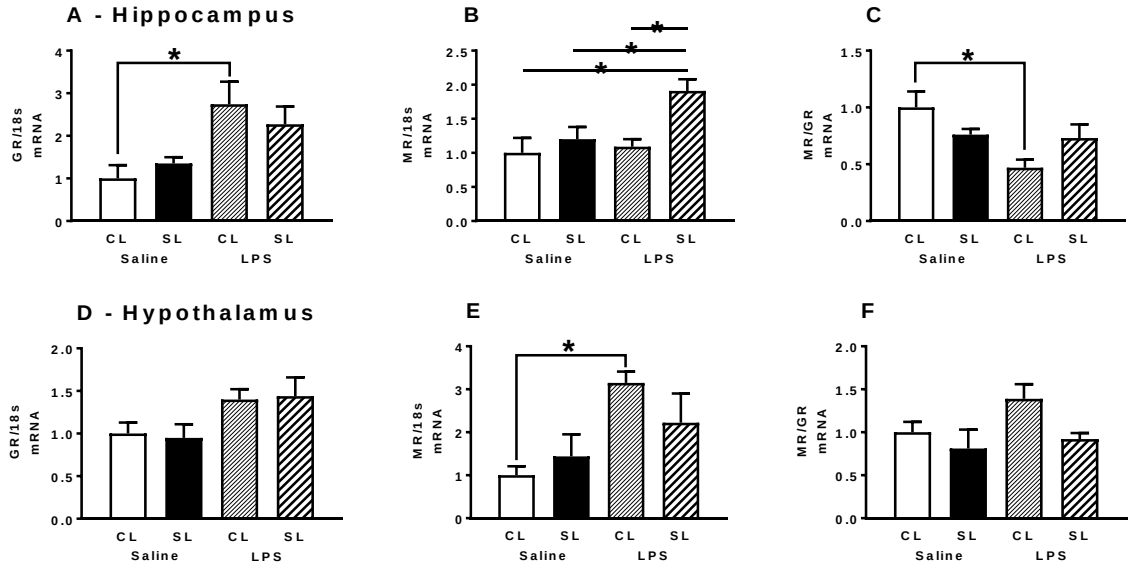


Figure 3.6 A – C) GR and MR gene expression in hippocampus 2 hours after LPS i.p. injection. D – F) GR and MR gene expression in hypothalamus 2 hours after LPS i.p. injection. A) Significant effect of litter size [$F_{(1,28)}$ = 12.29, p = 0.0016]. B) Significant effect of litter size [$F_{(1,28)}$ = 5.25, p = 0.0296] and LPS treatment [$F_{(1,28)}$ = 8.542, p = 0.0068]. C) Significant of interaction [$F_{(1,28)}$ = 6.039, p = 0.0205]. D) Significant effect of litter size [$F_{(1,28)}$ = 7.522, p = 0.0105. E) Significant effect of litter size [$F_{(1,28)}$ = 10.29, p = 0.0033]. F) Significant effect of LPS treatment [$F_{(1,28)}$ = 4.509, p = 0.0427]. *: $p < 0.05$, n = 8 animals per group. All data are mean + SEM.

3.4 Discussion

In this chapter, we have shown that early life diet has a significant long-term effect on the female rat as it does in the male. In Chapter 2, we showed male rats that were neonatally overfed have significant disruptions to their HPA axis responses to an immune challenge with LPS. Therefore, we aimed to investigate the long-term effects of neonatal overfeeding on HPA axis function in females here and we show the responses are not necessarily the same as those of the males.

As neonatal overfeeding caused the suppression of the male rats' HPA axis response to LPS, we hypothesised the LPS challenge would also affect the HPA axis of neonatally overfed female rats, but that there may be sex differences in these outcomes. From our group's previous studies, we have seen neonatal overfeeding leads to weight changes in both male and female rodents (Bulfin, Clarke et al. 2011, Clarke, Stefanidis et al. 2012, Spencer, Xu et al. 2012, Cai, Dinan et al. 2014). Our present experiment replicated this and yielded overweight adult female rodents to allow us to further analyse HPA axis function. From our male study, results showed enhanced Fos-positive cell numbers in the PVN after LPS (see Chapter 2). Thus, neonatally overfed male rats had approximately twice as many neurons activated in the PVN of the hypothalamus, as measured by immunohistochemistry for Fos-positive cells (Bulfin, Clarke et al. 2011). However, the same effect was not seen in females. In females, the same dose of LPS did not increase the number of neurons significantly activated in the PVN in any of the groups. The neonatal overfeeding response of the PVN to the immune challenge in females is thus not likely as sensitive as in males. These data are in contrast with previous work from our group comparing responses to restraint stress in neonatally overfed male and female rats. In this study, overfed female rats' PVN Fos-positive cell numbers were higher than controls after restraint, while the male response to restraint

stress was not affected by neonatal overfeeding (Spencer and Tilbrook 2009).

On the other hand, our present data from the females shows that the LPS-induced hippocampal MR gene expression in neonatally overfed females is greater than that of controls. Control female rats have a lower MR / GR ratio after LPS administration than under basal conditions, but the ratio did not change after stress in the neonatally overfed females. In the hypothalamus, the MR gene expression was increased after stress, however, this was not seen in neonatally overfed females. These data suggest neonatal overfeeding may alter the negative feedback of GC on the hippocampus and hypothalamus in female rats, whereas the pituitary and adrenal responses to LPS are not affected. No differences were seen in male MR or GR in the same brain regions (Chapter 2). In addition to over-nutrition in the neonatal period, obesity in adulthood can affect female MR and GR balance. When the MR / GR ratio is imbalanced, this can cause dysfunction of the HPA axis (de Kloet 2014). Thus, reduced levels of MR, but not GR, in the hippocampus, lead to increased HPA activity in obese female rats during restraint stress compared with controls (Mattsson, Lai et al. 2003). The dysregulation of GC negative feedback responses onto GR and MR in obese adult females can also impact the next generation by impacting maternal behaviour (Cottrell and Seckl 2009). In addition, Meaney et al.'s studies have shown stress from early life can change rodent and human GR gene expression in the hippocampus (Meaney and Aitken 1985, Francis and Meaney 1999, Turecki and Meaney 2016). From Boullu-Ciocca's studies, we see neonatal overfeeding can significantly increase PVN GR from P7 to P14, with GR expression normalising by P21. Moreover, proinflammatory cytokine over-expression may also change GR-dependent mechanisms (Boullu-Ciocca, Dutour et al. 2005, Boullu-Ciocca, Tassistro et al. 2015). The SL animals may also have more sensitive to restraint stress (Kenny, Dinan et al. 2014); stress may be induced by needle stick injury or the blood sampling procedure during experiment, then may be caused a significant increase in the expression of MR in the

hippocampus of SL animals 2 hours after LPS administration, compared to CL. The results also showed MR may be more important in regulating circardian fluctuations in corticosterone, with GR thought to be the driver of negative-feedback in response to stress-induced increases in corticosterone. Thus, neonatal overfeeding subtly alters HPA axis negative feedback, and this may leave these animals vulnerable to chronic stress or depression, subjects for future investigation (de Kloet, Oitzl et al. 1999, de Kloet, Karst et al. 2008, de Kloet 2014).

Previous studies showed the female sex hormones can delay the SAM and HPA axes responses to stress (Verma, Balhara et al. 2011). In ovariectomised rats, HPA axis responses to stress are similar to that of males, while in intact females HPA axis responses are larger, estradiol supports HPA axis stimulation (Stroud, Salovey et al. 2002). Similarly, testosterone suppresses HPA axis function (Viau and Meaney 1996, Rubinow, Roca et al. 2005). Thus, HPA axis signals (such as CRH, ACTH, etc.) are different in magnitude in response to stress between males and females, these differences are regulated by gonadal hormones (Goel, Workman et al. 2014). In addition, clinical studies have shown changes in the HPA axis activity can occur throughout the menstrual cycle (Goel, Workman et al. 2014). For example, during proestrus, basal ACTH and corticosterone levels are elevated (Figueiredo, Dolgas et al. 2002, Kudielka, von Kanel et al. 2006). Stress-induced cytokine secretion can also be different between men and women (Aulock, Deininger et al. 2006, Wegner, Benson et al. 2017), and between male and female animals (Crockett, Spielman et al. 2006, Hudson, Jacobson-Pick et al. 2014). One of the mechanisms is thought to be via progesterone, which alters the proinflammatory cytokine production (Miller and Hunt 1998, Goddard, Ton et al. 2013). We also need to pay attention that the human and animal HPA axis activity during the menstrual cycle is different, when we do the clinical studies (Abplanalp, Livingston et al. 1977, Verma, Balhara et al. 2011).

The MC2R is expressed in the cortex of the adrenal glands, and it is associated with MRAP to release GC (Hostinar, Sullivan et al. 2014, Dores and Garcia 2015). Based on the results of Chapter 2, we have seen neonatal overfeeding can lead to male rats responding to the immune challenge with a more suppressed MC2R gene expression than in controls, potentially leading to slower GC production. MRAP also tended to be suppressed in the neonatally overfed male rats relative to controls after LPS. The present female results show that although the MC2R gene expression had a tendency to be suppressed after LPS in the neonatally overfed, this was not significantly different and there were no differences in MRAP gene expression, indicating the slower GC release after LPS in neonatally overfed female rats is due to a different mechanism from that in males.

Previous work has shown nutrition restriction from late gestation can cause intrauterine fetal GC overexposure leading to growth delay, and modification of the HPA axis in the newborn pups. The GR and MR ratio in hippocampus but not hypothalamus was found to be suppressed in *in utero* nutrition-restricted pups. Also, GC-induced plasma ACTH levels were higher in nutritionally restricted dams' offspring, with MR reduced in the hippocampus, but GR unaffected (Lesage, Blondeau et al. 2001). Earlier studies have also shown stress-induced GC lead to increased MR relative to GR in the hippocampus and hypothalamus that was regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD)1 and 2 (Gomez-Sanchez and Gomez-Sanchez 2014). Thus, studies have used anti-glucocorticoid (RU38486) and anti-mineralocorticoid (RU28318) to inspect the effect on rats' plasma GC levels (Ratka, Sutanto et al. 1989). The results showed icv injection did not lead to increased plasma GC levels, but the GC levels were increased at 60 minutes after icv RU28318, returning back to normal after 90 minutes (Ratka, Sutanto et al. 1989). Such effects have also been shown in humans (Ladd, Huot et al. 2004). These findings seem to be true of females as well as males; thus, poor nutrition in dams can lead to long-term increases in the male and female offspring GR and

MR in the hippocampus in response to GC, as well as increases in plasma GC levels in response to stress (Cottrell and Seckl 2009, Xiong and Zhang 2013).

In conclusion, neonatal overfeeding influences female HPA axis long-term and the mechanism by which this occurs is likely different from males. In males, an insensitivity of the MC2R to LPS may account for slower GC release, but in females, the different MR / GR ratio at the hippocampus may be the main HPA axis change induced by neonatal overfeeding. The precise identification of the molecular mechanisms behind these changes remains to be discovered. However, neonatally overfed females may be less efficient in their response to bacterial endotoxin and the ability to respond to bacterial infection (Figure 3.7).

Figure 3.7 HPA axis functions in neonatally overfed female rat

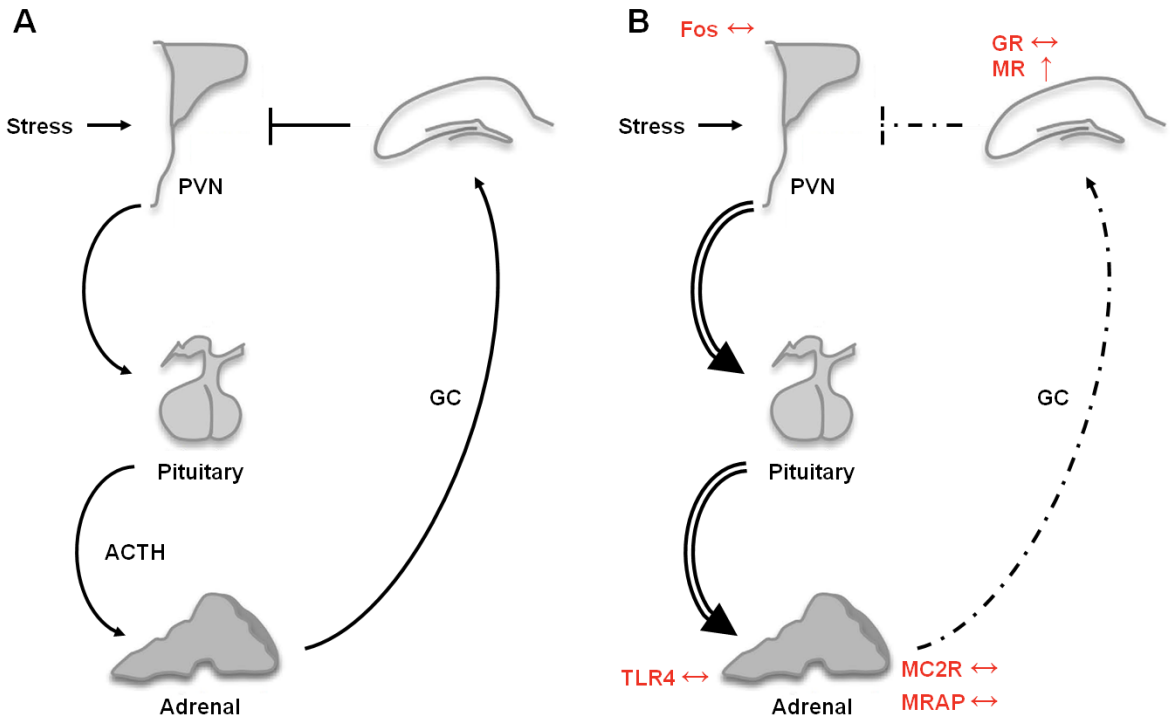
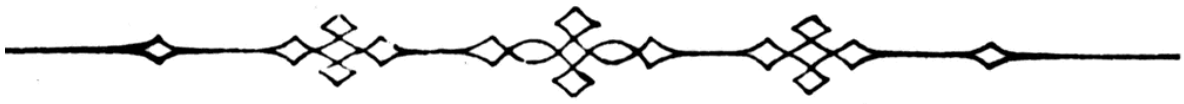


Figure 3.7 A) Under normal conditions, stress acts at the level of the brain to stimulate hypothalamic-pituitary-adrenal (HPA) axis activation and glucocorticoid (GC) production. GC feedback is central to suppress further HPA axis activation. B) In neonatally overfed female rats, central HPA axis responses to stress are likely to be normal, stimulating paraventricular nucleus of the hypothalamus (PVN) activation and adrenocorticotropic

hormone (ACTH) release from the pituitary. The melanocortin 2 receptor (MC2R) is not changed in the adrenals of neonatally overfed females. GC negative feedback and PVN neuronal activation seems normal. However, neonatal overfeeding changes the balance of GR to MR in females. Red arrows indicate the direction of gene differences between control and neonatally overfed groups after stress. GR, glucocorticoid receptor; MR, mineralocorticoid receptor; MRAP, melanocortin receptor accessory protein; POMC, pro-opiomelanocortin.



Chapter 4

Neonatal Overfeeding Attenuates Acute Central Pro-inflammatory Effects of Short-term High Fat Diet

These data have been published: “Guohui Cai, Tara Dinan, Joanne M. Barwood, Simone N. De Luca, Alita Soch, Ilvana Ziko, Stanley M.H. Chan, Xiao-Yi Zeng, Songpei Li, Juan Molero and Sarah J. Spencer (2015). Neonatal overfeeding attenuates acute central pro-inflammatory effects of short-term high fat diet. Front. Neurosci. 8, 446. doi: 10.3389/fnins.2014.00446.”, and a copy of the paper is included in the Appendix 2.

4.1 Introduction

As the introduction chapter describes, obesity in human childhood increases the risk of an individual becoming an obese adult, as well as increasing the risk of accompanying diseases such as diabetes, cardiovascular disease and stress (Whitaker, Wright et al. 1997, Stettler, Stallings et al. 2005, Biro and Wien 2010). In particular, as obese children mature, they are more likely to be affected by abnormal immune responses to inflammatory challenge as well as hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Reeves, Postolache et al. 2008, Lee 2009, Brune and Hochberg 2013). In animal studies, our group and other researchers have found rodent weight gain is significantly accelerated by neonatal overfeeding and this is associated with long-term effects on the immune system and HPA axis (Plagemann, Heidrich et al. 1992, Boullu-Ciocca, Dutour et al. 2005, Spencer and Tilbrook 2009, Clarke, Stefanidis et al. 2012, Smith and Spencer 2012, Stefanidis and Spencer 2012). In our previous experiments, we used lipopolysaccharide (LPS) to stimulate an immune response and saw the core body temperature response to LPS in neonatally overfed adults was significantly higher than in control rats, an effect that was evident in the juvenile period as well as adulthood (Clarke, Stefanidis et al. 2012). In accordance with this, there were more Fos-positive cells in the paraventricular nucleus (PVN) of hypothalamus after LPS in the neonatally overfed animals than the controls. These results indicate the HPA axis is more activated in response to LPS in the overfed animals (Clarke, Stefanidis et al. 2012).

Despite this evidence that neonatal overfeeding can influence weight gain and immune function long-term, some studies suggest not all overweight children become obese adults (Potter and Ulijaszek 2013) and not all overweight children or adults have immune disturbances (Oscai and McGarr 1978, Rodrigues, De Souza et al. 2007, Rodrigues, de Moura

et al. 2009). As such, our neonatally overfed rats have only mild changes in metabolic parameters (Stefanidis and Spencer 2012). In many cases of early life programming, a "double hit" or further stimulus is necessary later in life to bring out differences in the phenotype. For instance, Bilbo's papers have shown rats given neonatal immune challenge with *Escherichia coli* are normal in terms of peripheral cytokines and corticosterone under basal conditions but when given an LPS challenge as an adult, their cytokine responses are exacerbated (Bilbo, Biedenkapp et al. 2005, Bilbo and Schwarz 2009). Similarly, Walker et al.'s exposed neonatal rats to LPS and tested HPA axis and behavioural responses to stress. Under basal conditions, circulating corticosterone was elevated in the neonatally LPS-treated rats but the corticosterone response to repeated restraint was blunted and anxiety-like behavioural responses to LPS exacerbated (Walker, Nakamura et al. 2009). Moreover, inflammation and gliosis could permanently return to the mediobasal hypothalamus when give animal a long-term high fat diet (HFD) (Thaler, Yi et al. 2012). In addition, before the animals have the weight gain, the hypothalamic inflammatory neurons have already been activated to respond to the HFD within only 1 to 3 days. The reactive gliosis and markers proved the hypothalamic neuronal injury can occur after only 1 week HFD (Thaler, Yi et al. 2012). In this study, we hypothesised neonatal overfeeding would make animals more vulnerable to increased weight gain and immune dysfunction when the rodents' accelerated metabolic responses are subjected to the "double hit" of a 3 days or 3 weeks high fat diet (HFD) commenced in adulthood.

In these studies, we used the same animal model as described in previous chapters. We thus manipulated the litter sizes the newborn rats were suckled in, creating litters of 4 (small litter; neonatal overfeeding; SL) or 12 (control litter; CL). Then, when the pups reached adulthood, we gave them either 3 days or 3 weeks HFD (23.5% fat, 45% kilocalories as fat) and compared responses with normal chow (4.8% fat, 12% kilocalories as fat)

controls. At the end of the high fat feeding period, we measured changes in weight gain and indices of diabetes including responses to a glucose tolerance test. We also analysed central and peripheral markers of inflammation in response to intraperitoneal (i.p.) LPS injection.

4.2 Methods and materials

The methods and materials are as described in Chapter 2, except where is noted below. All experiments were initiated between 0900 and 1200 hour to limit potential effects of circadian rhythms on any parameters measured. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals. All these were approved by the RMIT University Animal Ethics Committee (see Appendix 3).

4.2.1 Animals

Litter size manipulation was conducted as described in Chapter 2, and there were 88 Wistar male rats and 80 Wistar female rats involved. In order to test neonatal overfeeding effects on the rats' long-term susceptibility to HFD, we allocated the rats into same-sex littermate pairs and put them on *ad libitum* normal rat chow from postnatal day (P)21 to P56. At P56 the rats were changed to the 3 days or 3 weeks HFD (23.5% fat; 45% kilocalories from fat; Specialty Feeds, WA, Australia) as the introduction described, or normal rat chow diet (4.8% fat; 12% kilocalories as fat; Specialty Feeds, WA, Australia), the food intake of every pair was recorded for energy consumption calculation every day for 3 days and 3 weeks. 3 days HFD was begun at P74 and 3W HFD (as above) was set up at P56 so that the rats were the same age when culled.

4.2.2 Glucose tolerance test (GTT) and plasma triglycerides

On P76, after 2 days or 20 days high fat feeding or equivalent in chow fed controls, we gave the rats an i.p. glucose tolerance test (GTT) and collected blood for plasma triglycerides. Before testing basal glucose levels, rats were fasted for 3 – 4 hours. We then

quickly took each rat from its cage and nicked the tip of the tail with a sharp razor blade to collect approximately 20 µL of baseline blood sample into a heparinised capillary tube for measurement of plasma triglycerides. Blood plasma and liver triglycerides were later measured by colorimetric enzymatic GPO-PAP assays (Roche Diagnostics, Indianapolis, Indiana, USA). Blood samples were kept on ice until the end of the experiment when they were centrifuged and the plasma aliquots stored at -20 ^{o}C until assayed. We also detected basal glucose levels at this time using an Accu-Chek Performa blood glucose meter (Roche Diagnostics; Castle Hill, New South Wales, Australia). We then gave each rat an i.p. injection of 1.5 g/kg glucose and analysed glucose levels with the Accu-Chek glucose meter at 15, 30, 45, 60, and 90 minute after glucose i.p. injection.

4.2.3 Immune challenge and tissue collection

Two days after GTT (i.e. after 4 or 22 days HFD or chow diet feeding), the pairs of rats were then randomly allocated into saline or LPS groups. We gave each rat an i.p. injection of LPS or pyrogen-free saline as in Chapter 2. At 2 hours after LPS injection, we deeply anaesthetized the rats with Lethabarb. We hemisected each rat below the diaphragm and used it for fresh tissue collection and for cardiac perfusion to obtain fixed brains. Also, we removed livers and male epididymal or female perirenal fat pads. Tissues were weighed and snap-frozen in liquid nitrogen immediately. Brain tissues were processed for immunohistochemistry for immune activated marker c-Fos as detailed in Chapter 2.

4.2.4 Inflammatory gene expression

We use same methods and materials are as described in Chapter 2 to assess changes in peripheral markers of inflammation cytokines. We measured mRNA expression levels of LPS receptor toll-like receptor (TLR)4 and its downstream transcription factor nuclear factor

κB (NFκB), as well as representative pro- and anti-inflammatory cytokines, interleukin (IL)-10, tumour necrosis factor (TNF)α, IL-1β, and IL-6 in the liver and adipose tissues.

Table 4.1 Liver cytokine responses to LPS after 3 days and 3 weeks HFD

Target Gene	NCBI Reference Sequence	TaqMan Assay ID	Amplicon Length
Nfkb1	NM_001276711.1	Rn01399572_m1	67
Il10	NM_012854.2	Rn01483988_g1	105
Tnf	NM_012675.3	Rn00562055_m1	82
Il1b	NM_031512.2	Rn00580432_m1	74
Il6	NM_012589.2	Rn01410330_m1	121
Tlr4	NM_019178.1	Rn00569848_m1	127
18s	X03205.1	4319413E	187

4.2.5 Cell counts

An experimenter, blinded to the group treatments, also carried out counts of cells positive for Fos-immunoreactivity in the PVN over two sections (~1.80 and 1.95 mm caudal to bregma), in the dorsal (d) and ventral (v) bed nucleus of the stria terminalis (BNST) over four sections (~0.24 to −0.36 mm relative to bregma), in the medial preoptic area (MPOA) and vascular organ of the laminar terminalis (OVLT) over two sections (~0.36 and 0.51 mm rostral to bregma) and in the ventromedial (VM) POA over two sections (at and 0.15 mm caudal to bregma).

4.2.6 Liver Cytokine Expression

To further assess changes in peripheral markers of inflammation, we examined concentrations of a number of pro- and anti-inflammatory cytokines in the liver using a Bio-Plex assay allowing multiple analytes to be assessed in one sample. Liver samples were lysed using Bio-Plex cell lysis kit (Bio-Rad) according to the manufacturer's instructions. The total

protein concentration of the lysates was determined using the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific). Samples were then diluted in Bio-Plex Sample Diluent (containing 0.5% BSA) and assayed in a final concentration of 500 ug/mL using a magnetic beads-based Bio-Plex Pro rat TH1/TH2 12-Plex (Bio-Rad) assay. The assays were performed using the Bio-Plex MAGPIX™ instrument and the data were analyzed using Bio-Plex Manager Software 6.1 (Bio-Rad). Female IL-13, granulocyte macrophage colony-simulating factor, and interferon gamma were not detectable and these were low and not significantly different between groups in the males, so are not reported here.

4.2.7 Data analysis

IBM SPSS 22 was used for statistical analyses and GraphPad was used for preparation of graphs. To compare pre-weaning body weights between CL and SL rats, an analysis of variance (ANOVA) with repeated measures was used, with litter size as the between factor and age as the repeated measure. When a significant interaction was found between litter size and age, Student's unpaired t-tests were performed for each time point. Since a significant effect of age on weight was expected and not the primary subject of our investigation, we made an *a priori* decision to limit our individual comparisons to the effect of litter size. Thus, once a significant interaction between age and litter size was found, the appropriate comparisons were between the two litter size groups at each age, so only t-tests are necessary. Adult parameters were compared using multi-factorial ANOVAs with litter size, sex, adult diet, and LPS treatment as between factors where appropriate, with Tukey's *post hoc* comparisons where significant main effects or interactions were found. We also included time (minutes) as a repeated measure in analysis of plasma corticosterone concentrations. Data are presented as the mean + standard error of the mean (SEM). Statistical significance was assumed when $p < 0.05$.

4.3 Results

4.3.1 Neonatal overfeeding significantly increased body weight in the neonatal period and adulthood

As previously seen (Chapter 2), compared with rats from CL, the neonatal overfeeding lead to body weight gain during suckling (Figure 4.1A) and into adulthood (Figure 4.1B).

Figure 4.1 Effects of neonatal overfeeding on body weight

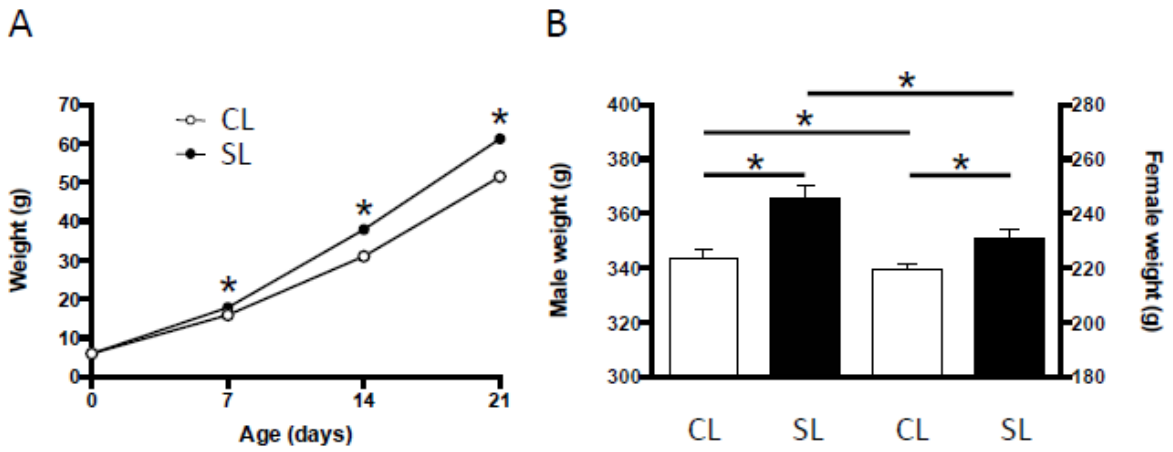


Figure 4.1 A) Pre-weaning total body weights of rats raised in control (CL) and small (SL) litters. Pups were weighed in whole litter units every 7 days until weaning. Significant age, litter size interaction [$F_{(3, 78)} = 28.83$, $p < 0.001$]. *: $p < 0.05$. n = 8 litters per group. B) Adult body weights. Significant effect of litter size [$F_{(1, 84)} = 25.96$, $p < 0.001$] and sex [$F_{(1, 84)} = 1559.46$, $p < 0.001$]. *: $p < 0.05$. n = 8 animals per group. All data are mean + SEM.

4.3.2 Weight gain, food intake, and caloric efficiency with HFD in adulthood

Neonatal overfeeding did not induce significant differences in the weight gained with the 3 days HFD in males or females (Figure 4.2A and B). There were significant effects of sex and diet, with females gaining less weight over the period than males, and those on HFD gaining less weight than those on standard rat chow, but there were no differences between relevant groups with *post hoc* comparisons. After 3 weeks of HFD, all female groups had gained less weight than all male groups. There was also an effect of litter size, with SL gaining more weight than CL but no differences between relevant groups with *post hoc* comparisons (Figure 4.2C and D).

Consistent with their size, females ate less than males in both the 3 days and 3 weeks analyses. There was also a significant effect of diet on food intake after 3 weeks, with HFD-fed rats eating fewer grams of food than standard chow-fed rats, in total and for each of the 3 weeks (Figure 4.3A, B, C, and D).

Calculations of total energy consumption revealed the HFD groups consumed more energy than the chow groups at 3 days and 3 weeks, and males consumed more energy than females. However, there was no influence of neonatal overfeeding on total energy consumption (Figure 4.4A, B, C, and D).

Caloric efficiency is a measure of the ability to convert calories into body weight. Thus, a reduced caloric efficiency reflects the need to consume more calories to maintain body weight. 3 days HFD significantly reduced caloric efficiency in SL but not CL male and female rats (Figure 4.5A and B). The 3 weeks HFD significantly reduced the caloric efficiency in SL but not CL females (Figure 4.5C and D).

Figure 4.2 Weight gain after HFD

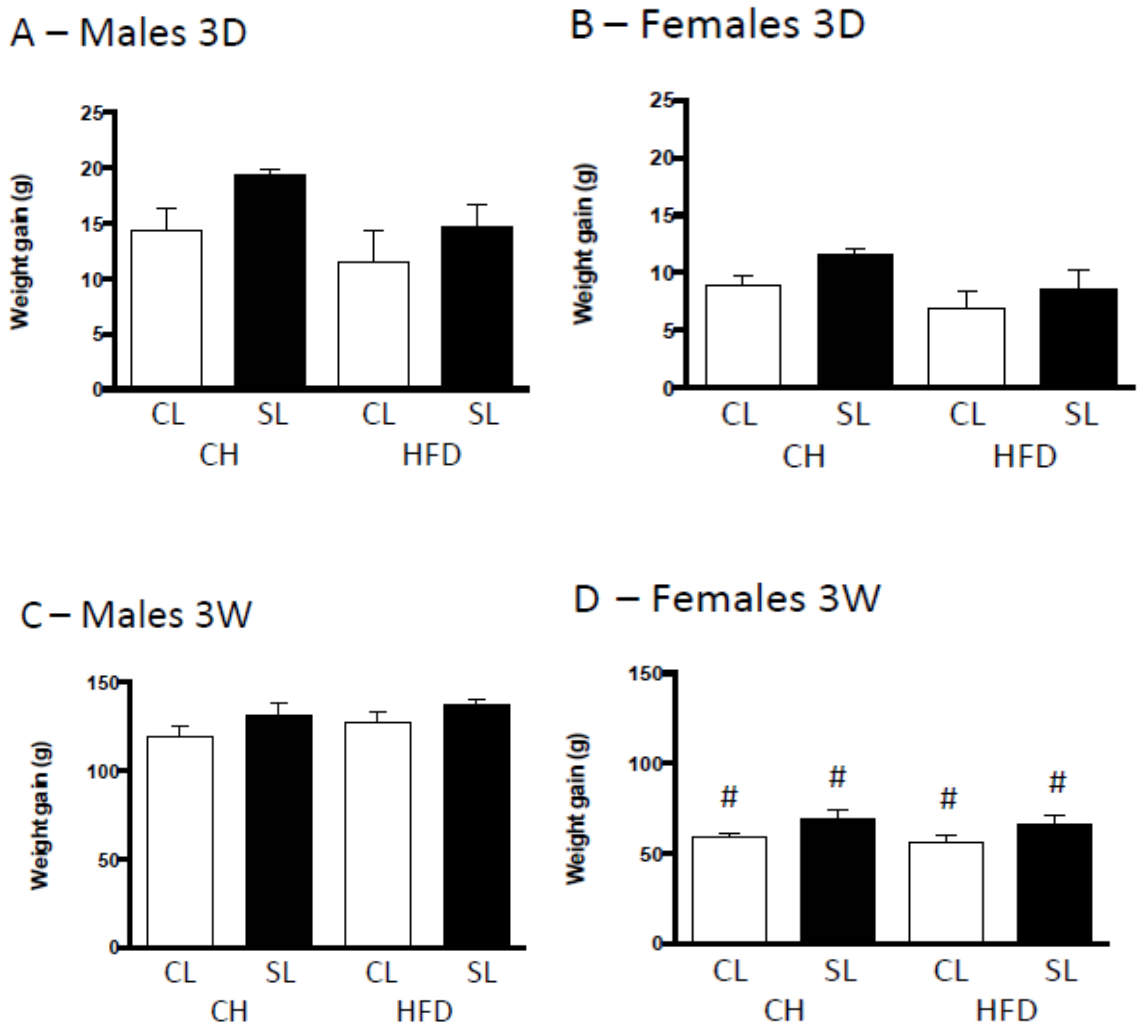


Figure 4.2 Effects of normal (control, CL) and neonatal overfeeding (small litter, SL) in male and female rats on weight gain, and food intake after 3 days (3D) or 3 weeks (3W) high fat diet (HFD) or chow (CH). Weight gain with 3D (A and B) and 3W (C and D) HFD or CH in male (A and C) and female (B and D) adult rats from in CL and SL. 3D HFD: significant effect of sex [$F_{(1, 48)}$ = 26.77, p < 0.001] and diet [$F_{(1, 48)}$ = 7.16, p = 0.01]. 3W HFD: significant effect of sex [$F_{(1, 50)}$ = 374.35, p < 0.001] and litter size [$F_{(1, 50)}$ = 9.78, p = 0.003]. #: Sex difference between corresponding groups, p < 0.05, n = 8 animals per group. All data are mean + SEM.

Figure 4.3 Food intake in 3 days and 3 weeks HFD

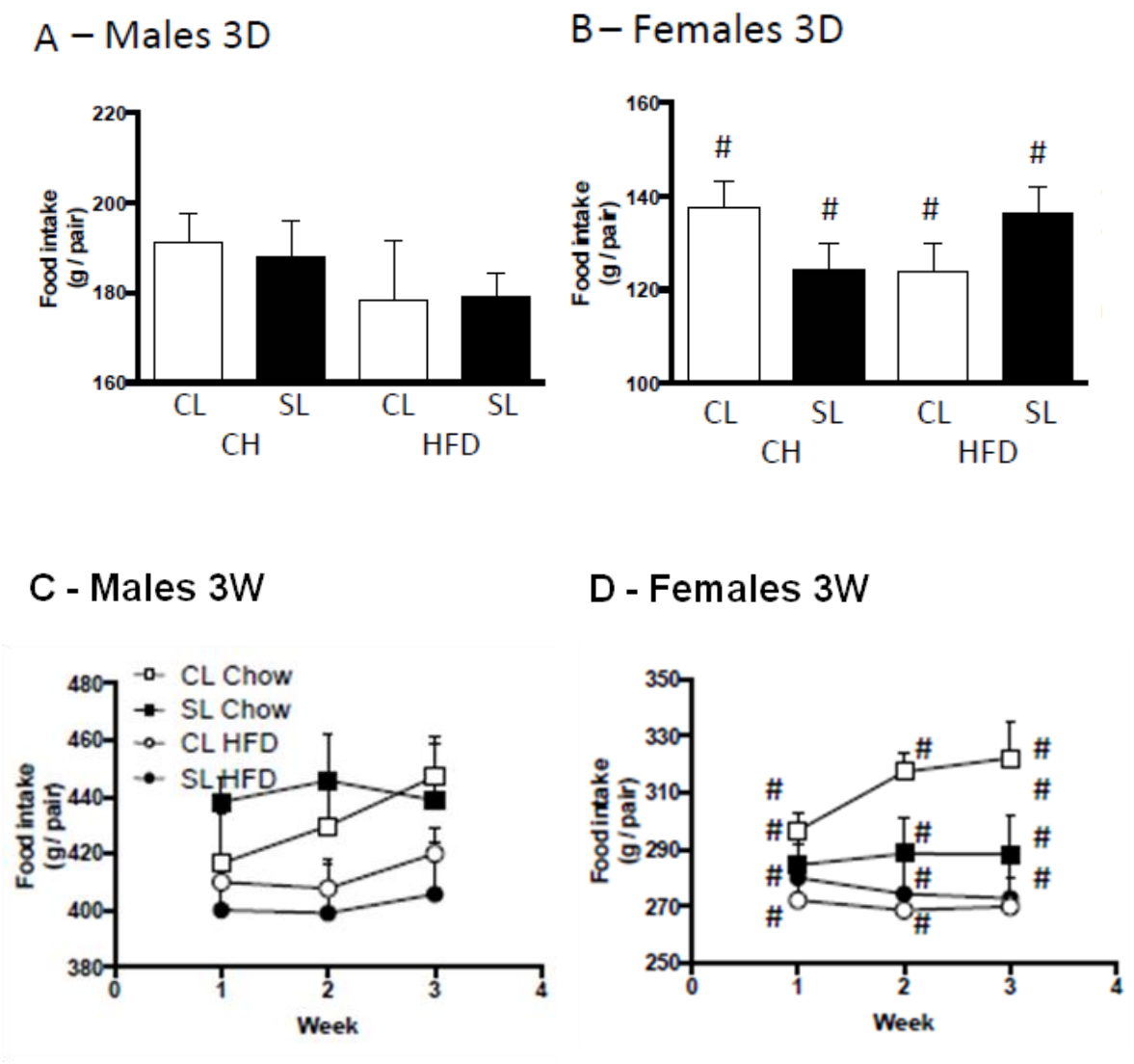


Figure 4.3 Food intake with 3 days (3D; A and B) and 3 weeks (3W; C and D) high fat diet (HFD) or chow (CH) in males (A and C) and females (B and D). 3D HFD: significant effect of sex [$F_{(7, 48)} = 110.74$, $p < 0.001$]. 3W HFD: significant effect of sex [$F_{(7, 46)} = 285.23$, $p < 0.001$] and diet [$F_{(7, 46)} = 12.03$, $p = 0.001$]. #: Sex difference between corresponding groups, $p < 0.05$, n = 7 animals per group. All data are mean + SEM.

Figure 4.4 Energy intake in 3 days and 3 weeks HFD

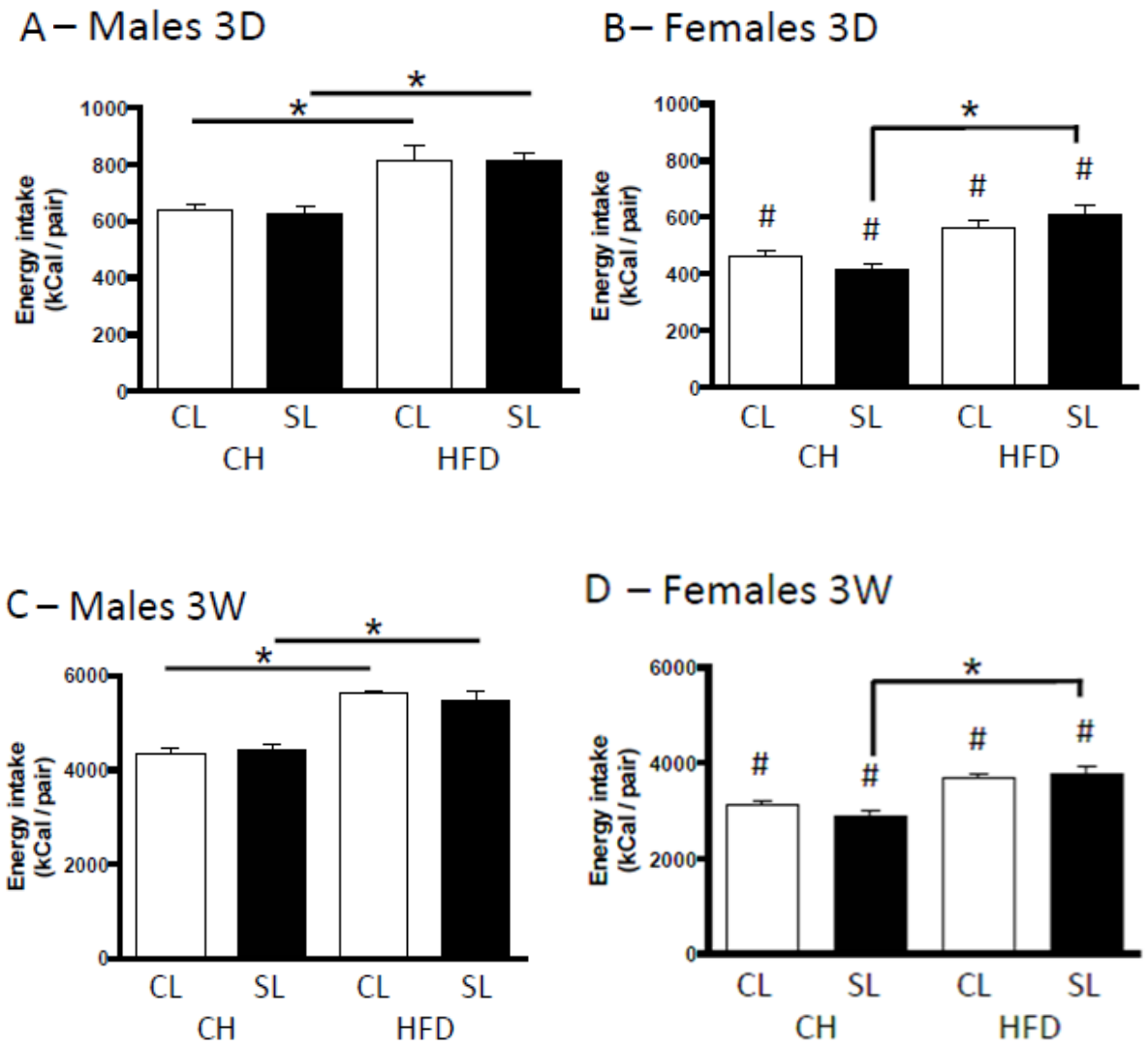


Figure 4.4 Energy intake with 3 days (3D; A and B) and 3 weeks (3W; C and D) high fat diet (HFD) or chow (CH) in males (A and C) and females (B and D). 3D HFD: significant effect of sex [$F_{(7, 45)} = 88.54$, $p < 0.001$], and diet [$F_{(7, 45)} = 53.58$, $p < 0.001$]. 3W HFD: significant effect of sex [$F_{(7, 46)} = 266.19$, $p < 0.001$], and diet [$F_{(7, 46)} = 93.34$, $p < 0.001$], significant sex × diet interaction [$F_{(7, 46)} = 5.54$, $p = 0.023$]. #: Sex difference between corresponding groups. *: $p < 0.05$, n = 8 animals per group. All data are mean + SEM.

Figure 4.5 Caloric efficiency in 3 days and 3 weeks HFD

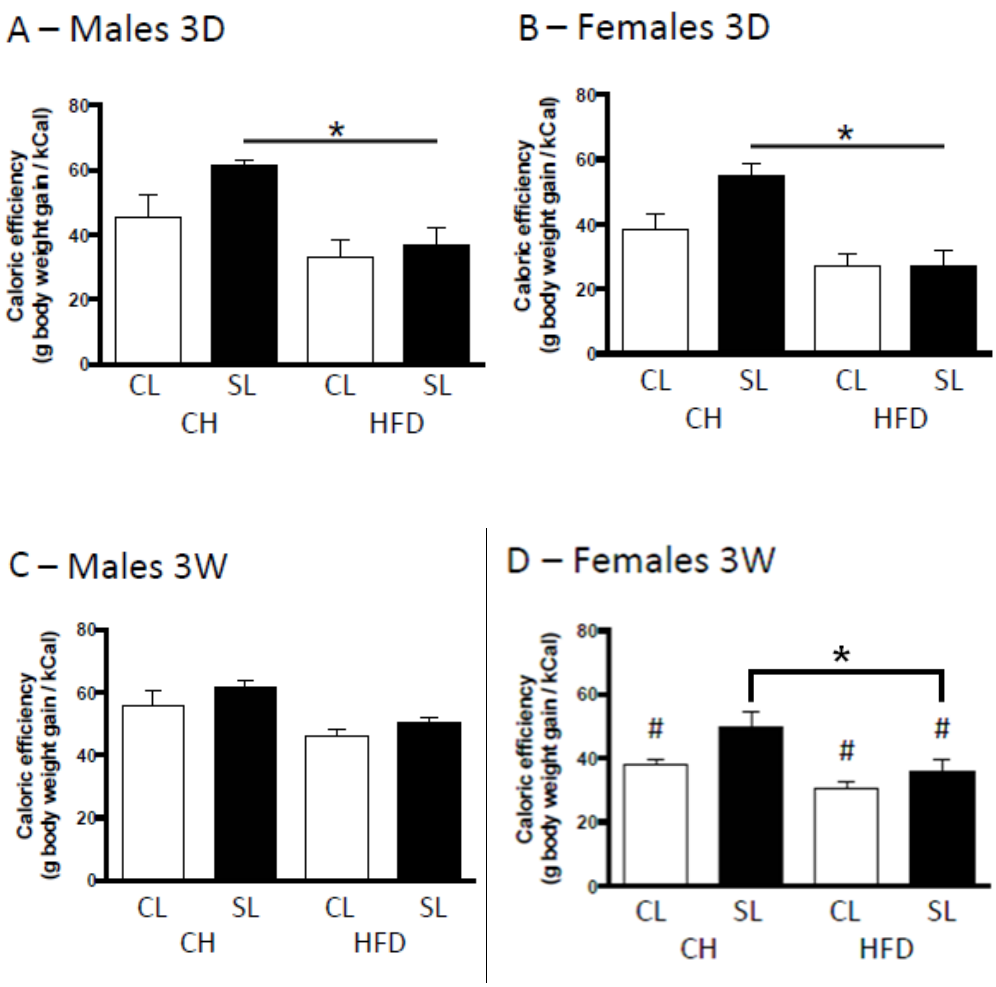


Figure 4.5 Caloric efficiency with 3 days (3D; A and B) and 3 weeks (3W; C and D) high fat diet (HFD) or chow (CH) in males (A and C) and females (B and D). 3D HFD: significant effect of litter size [$F_{(7, 45)}$ = 6.83, p = 0.012], sex [$F_{(7, 45)}$ = 4.53, p = 0.039], and diet [$F_{(7, 45)}$ = 30.73, p < 0.001], significant litter size × diet interaction [$F_{(7, 45)}$ = 4.45, p = 0.041]. 3W HFD: significant effect of litter size [$F_{(7, 46)}$ = 10.75, p = 0.002], sex [$F_{(7, 46)}$ = 51.85, p < 0.001], and diet [$F_{(7, 46)}$ = 25.39, p < 0.001]. When we compared males and females together, there was a significant effect of three week HFD diet in both male CL and SL groups and female SL group, however there were no significant diet effect in female CL group. #: Sex difference between corresponding groups. *: p < 0.05, n = 8 animals per group. Data are mean + SEM.

4.3.3 Fat mass and triglyceride content with HFD in adulthood

Surprisingly, there were no differences in total or percentage fat between any of the CL and SL groups (Figure 4.6A, B, C, and D). We did not make a sex comparison in this analysis since the fat pads were different. There was a significant effect of litter size on plasma triglyceride concentrations, with generally increased triglyceride levels in rats from SL. There was also an effect of sex, with females of each group having lower triglyceride levels than their male counterparts (Figure 4.7A and B). We also detected significant effects of litter size and diet on liver triglyceride concentrations, with SL and the HFD increasing these levels (Figure 4.7C and D).

Figure 4.6 Fat mass in neonatally overfed animal after 3 days or 3 weeks HFD

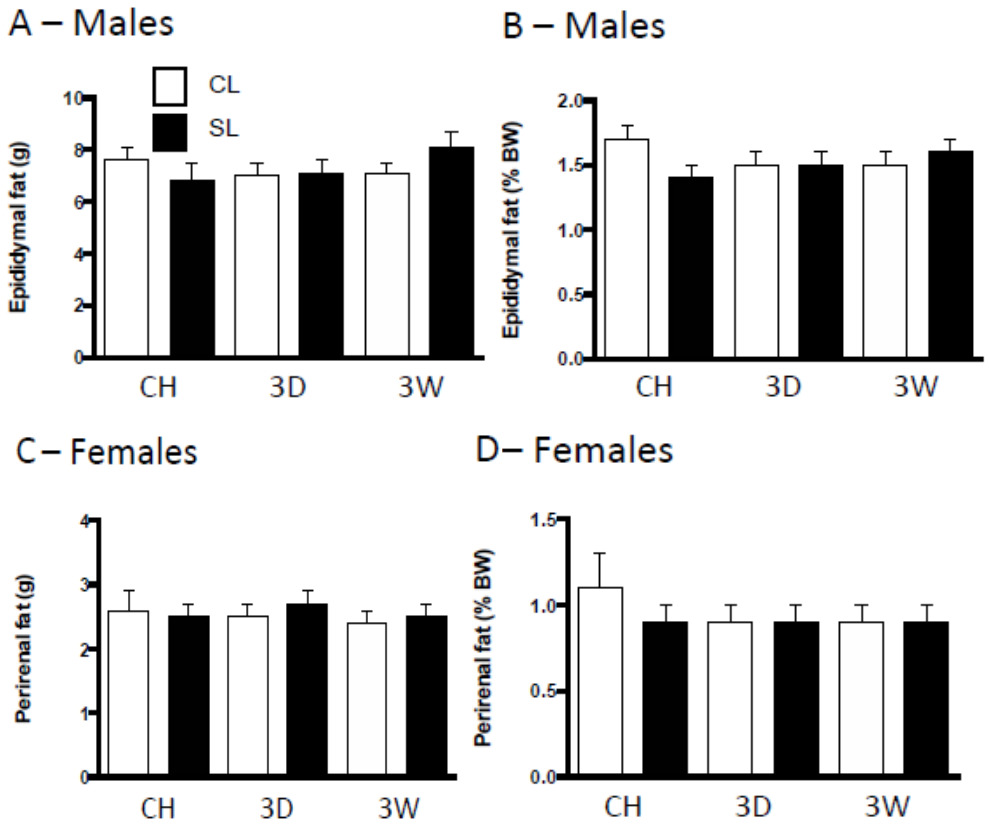


Figure 4.6 Total fat (A and C) and percentage fat (B and D) after 3 days (3D) and 3 weeks (3W) HFD or chow (CH) in male (A and B) and female (C and D) adult rats were raised in control (CL) and small (SL) litters. n = 8 animals per group. Data are mean + SEM. There were no differences between groups.

Figure 4.7 Triglyceride content in neonatally overfed animal after 3 days or 3 weeks HFD

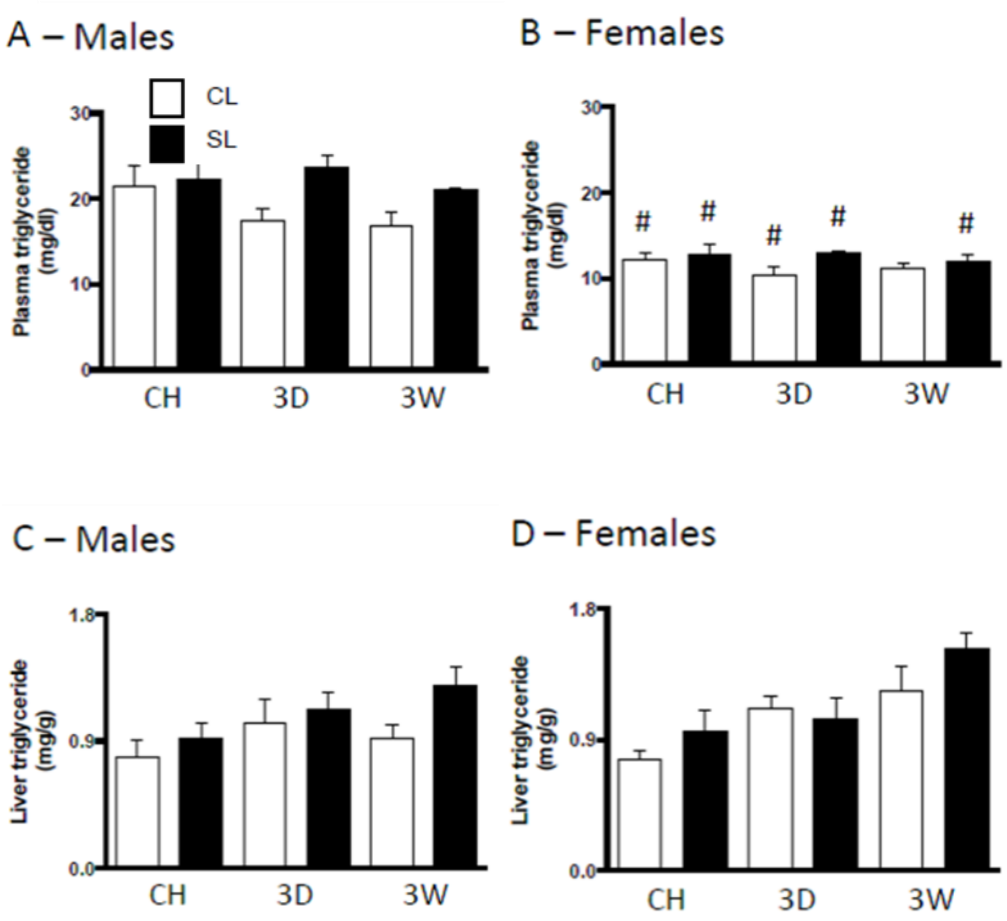


Figure 4.7 Plasma triglycerides in males (A) and females (B) after 3 days (3D) or 3 weeks (3W) HFD or chow (CH). Significant effect of litter size [$F_{(11, 91)} = 4.64$, $p = 0.034$], and sex [$F_{(11, 91)} = 81.91$, $p < 0.001$]. C and D) Liver triglycerides in males (C) and females (D) after 3Dor 3W HFD or CH. Significant effect of litter size [$F_{(11, 72)} = 4.24$, $p = 0.043$] and diet [$F_{(11, 72)} = 5.69$, $p = 0.001$]. #: Sex difference between corresponding groups, $p < 0.05$, n = 8 animals per group. Data are mean + SEM.

4.3.4 Glucose utilisation with HFD in adulthood

In accordance with the minimal effects of the HFD seen on overt measures of weight gain and adiposity, we also saw no significant differences in fasting glucose levels, or tolerance to glucose among the groups in males or females (Figure 4.8A, B, C, and D).

Figure 4.8 Glucose utilisation in neonatally overfed animal after 3D and 3W HFD

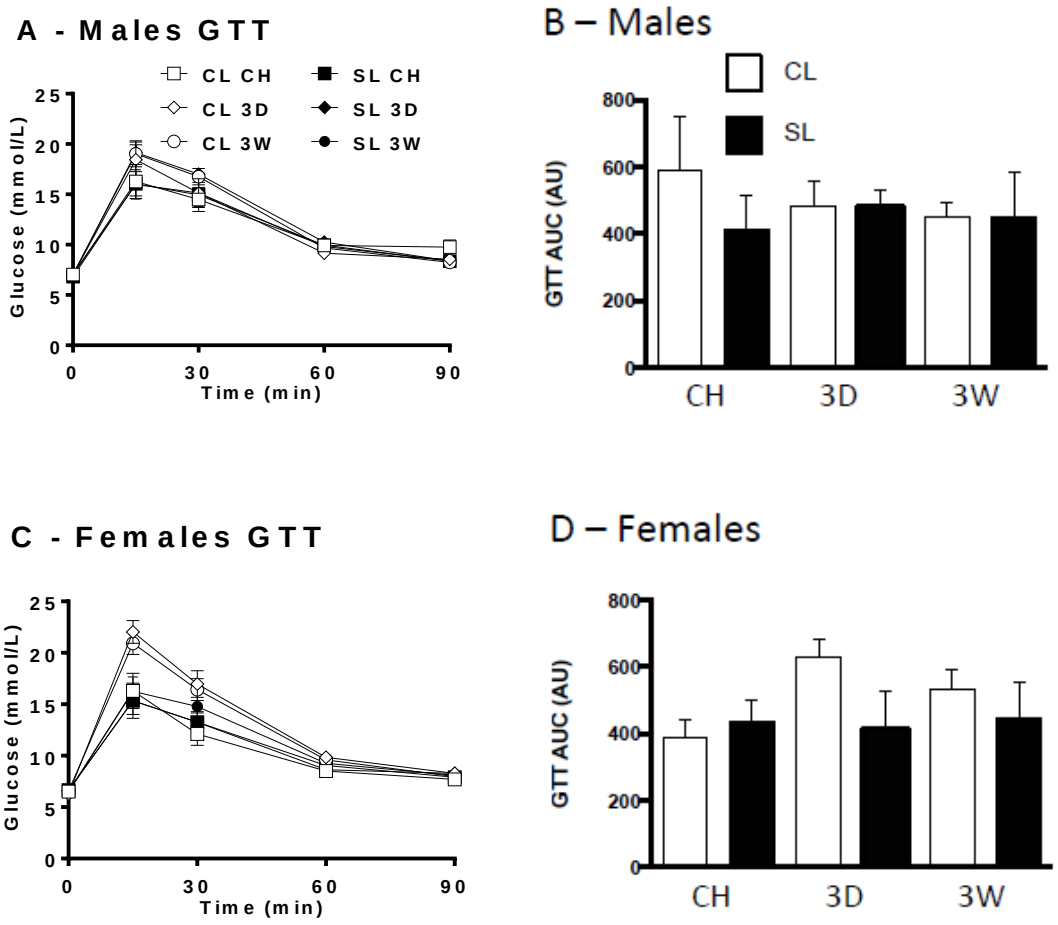


Figure 4.8 Effects of neonatal overfeeding on glucose utilisation after 3 days (3D) or 3 weeks (3W) HFD or chow (CH). Glucose concentrations (A and C) and incremental area under the curve (iAUC) responses (B and D) to an i.p. glucose tolerance test after 3D and 3W HFD or CH in male (A and B) and female (C and D) adult rats were raised in control (CL) and small (SL) litters. n value for male is CL/CH: 6, CL/3D: 6, CL/3W: 8, SL/CH: 7, SL/3D: 8, SL/3W: 8 animals; female is CL/CH: 7, CL/3D: 8, CL/3W: 8, SL/CH: 7, SL/3D: 8, SL/3W: 8 animals. Data are mean + SEM. There were no differences between groups.

4.3.5 Peripheral inflammation with HFD in adulthood; gene expression

We have previously reported neonatal overfeeding influences peripheral and central immune profiles (Clarke, Stefanidis et al. 2012, Ziko, De Luca et al. 2014). Therefore, we tested if neonatal overfeeding exacerbates the peripheral and central response of inflammatory markers to HFD. In the liver, there was an increase in TLR4 mRNA after 3 days HFD in both CL and SL males compared with their chow-fed counterparts. Interestingly, this increase in TLR4 did not persist but had returned towards baseline values after 3 weeks (Figure 4.9A). There were no significant differences between the female groups with *post hoc* tests and no sex differences, but CL females did show a tendency to have elevated TLR4 after 3 days HFD compared with chow-fed females (Figure 4.9B).

Figure 4.9 TLR4 gene expression in neonatally overfed rat liver after 3D and 3W HFD

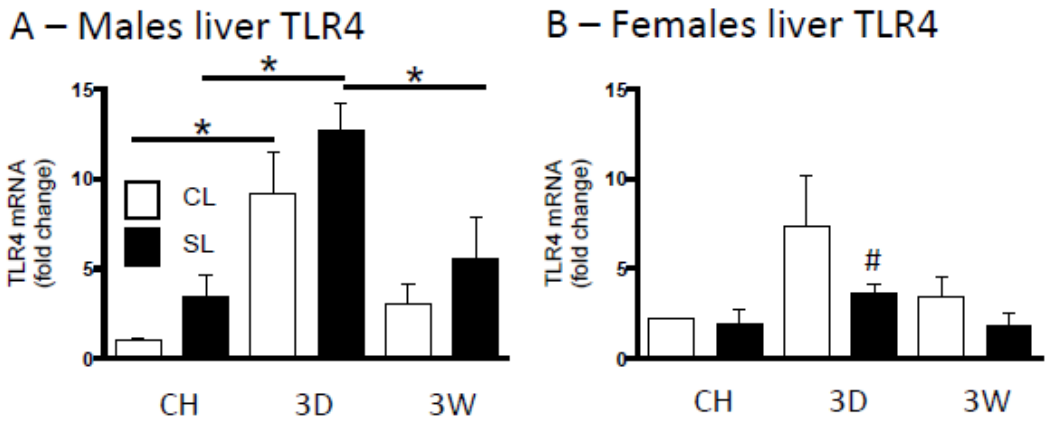


Figure 4.9 Effects of neonatal overfeeding on peripheral inflammatory gene expression after 3 days (3D) or 3 weeks (3W) HFD or chow (CH). Liver TLR4 (A and B) after 3D and 3W HFD or CH in male and female adult rats were raised in control (CL) and small (SL) litters. Significant effect of diet [$F_{(11, 60)} = 18.71$, $p < 0.001$] and sex [$F_{(11, 60)} = 8.25$, $p = 0.006$], significant litter size × sex interaction [$F_{(11, 60)} = 7.47$, $p = 0.008$], significant diet × sex interaction [$F_{(11, 60)} = 3.39$, $p = 0.04$]. *: $p < 0.05$, n = 8 animals per group. #: Sex difference between corresponding groups. Data are mean + SEM.

In liver, there was a significant effect of sex on NFκB, IL-10, and IL-1β mRNA, with females expressing more of these three genes than males, but there were no significant differences with *post hoc* tests except in that there was more IL-1β in females after 3 weeks HFD than in males. There were no differences between the groups in liver TNFα mRNA and IL-6 was undetectable in this tissue (Figure 4.10A, B, C, and D).

Figure 4.10 Neonatally overfed rat liver peripheral inflammatory gene expression

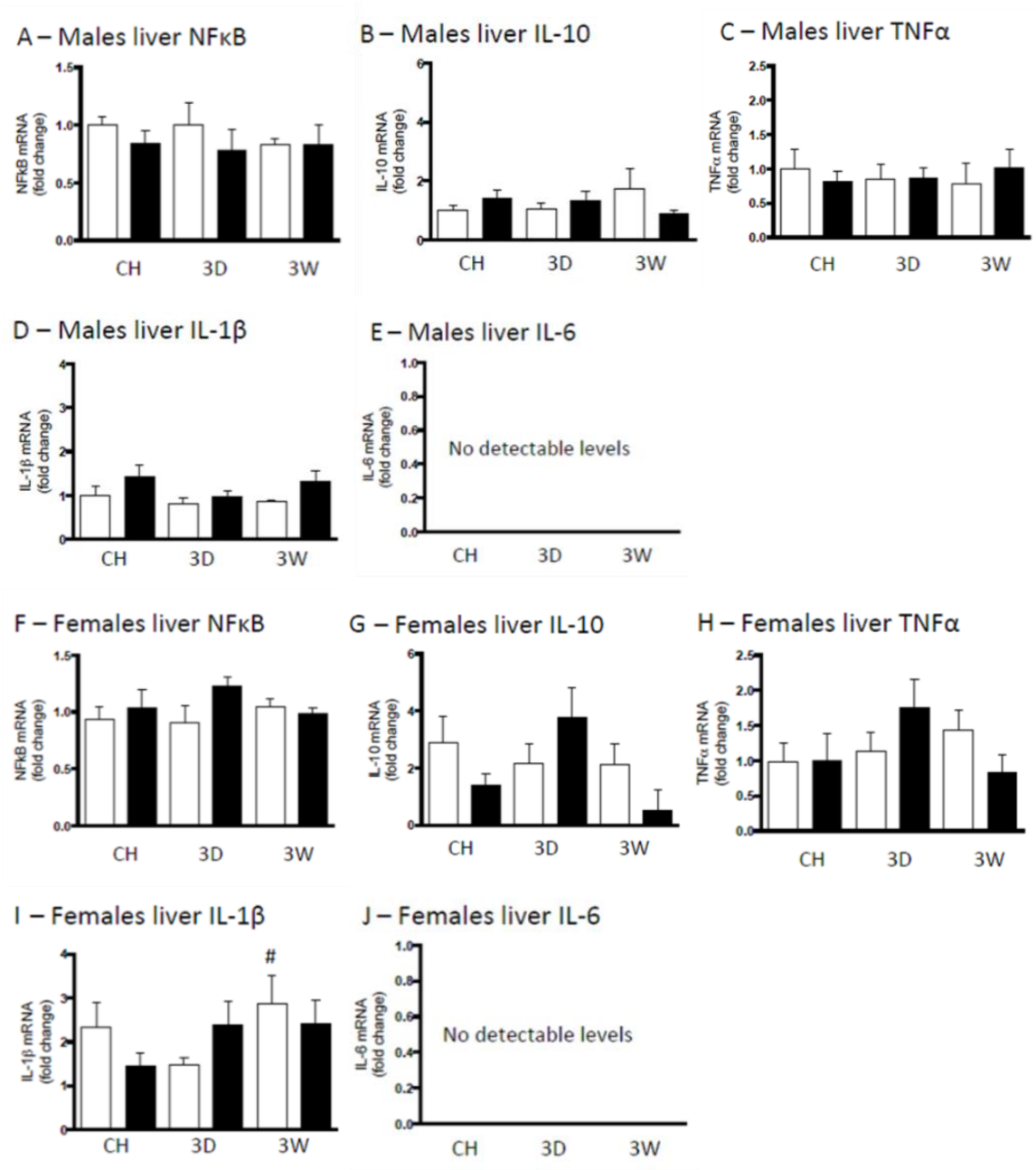


Figure 4.10 A – E) Effects of neonatal overfeeding in male rats on peripheral inflammatory gene expression after 3 days (3D) or 3 weeks (3W) HFD or chow (CH). F – J) Effects of neonatal overfeeding in female rats on peripheral inflammatory gene expression after 3D or 3W HFD or chow. Nuclear factor κB (NFκB), interleukin (IL)-10, tumour necrosis factor (TNF)α, IL-1β and IL-6 after 3D and 3W HFD or CH in male and female adult rats that were raised in control (CL) and small (SL) litters. Liver NFκB: significant effect of sex [$F_{(11, 56)}$ = 4.12, p = 0.047]. Liver IL-10 significant effect of sex [$F_{(11, 57)}$ = 11.25, p = 0.001]. Liver: IL-1β significant effect of sex [$F_{(11, 59)}$ = 26.42, p < 0.001]. #: Sex difference between corresponding groups. n = 8 animals per group. Data are mean + SEM.

We analysed male epididymal and female perirenal fat separately as the fat was taken from different regions. There was a significant effect of litter size on fat NFκB in the males, with SL having more NFκB than CL, but there were no significant differences between the individual groups with *post hoc* tests on male fat NFκB. There was also a significant effect of diet on male IL-10 and IL-1β with the HFD reducing expression of these cytokines, but again there were no *post hoc* differences and no further significant differences in male or female fat TLR4, NFκB, TNFα, or IL-6 mRNA (Figure 4.11A, B, C, and D).

4.3.6 Peripheral inflammation with HFD and LPS in Adulthood; liver protein

Analysis of liver concentrations of a suite of pro- and anti- inflammatory cytokines revealed no notable effects of HFD at 3 days or 3 weeks in any of the groups and no notable effects of the litter size except where IL-2 was suppressed in overfed rats relative to controls. LPS injection significantly increased liver IL-1α, IL-1β, IL-6, and TNFα across the groups, but there were no significant differences with the *post hoc* tests, except in IL-1α controls after 3 days HFD. We also found significant sex differences, with less of all the cytokines measured in females than in males, except IL-1α (Table 4.2).

Figure 4.11 Neonatally overfed rat fat peripheral inflammatory gene expression

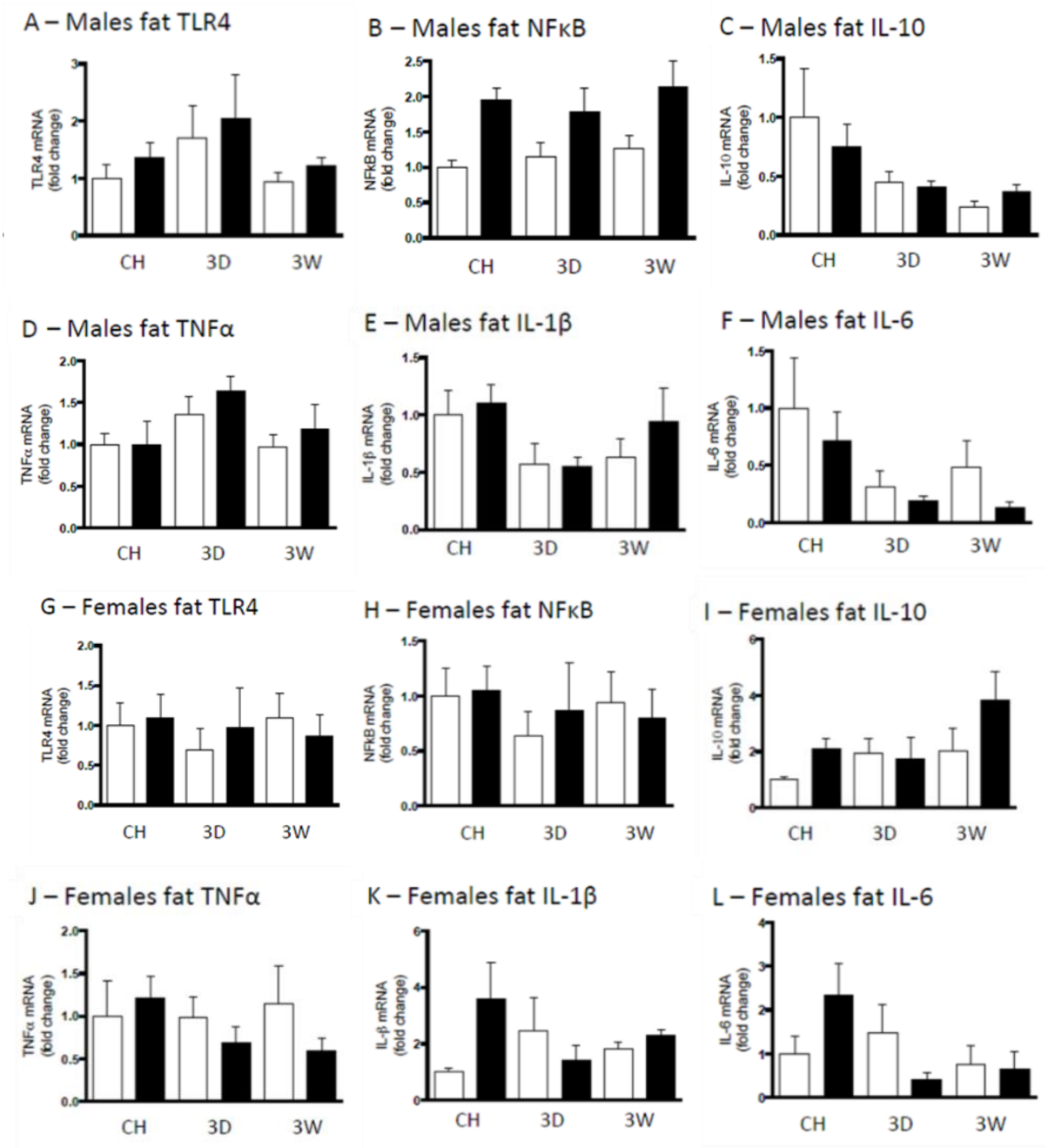


Figure 4.11 A – F) Effects of neonatal overfeeding in male rats on peripheral inflammatory gene expression after 3 days (3D) or 3 weeks (3W) HFD or chow (CH). G – L) Effects of neonatal overfeeding in female rats on peripheral inflammatory gene expression after 3D or 3W HFD or chow. NFκB, interleukin (IL)-10, TNFα, IL-1β and IL-6 after 3D and 3W HFD or CH in male and female adult rats that were raised in control (CL) and small (SL) litters. Fat TLR4 gene expressions after 3D and 3W HFD or CH in adult male or female rats were raised in control and small litters. Male fat NFκB: significant effect of litter size [$F_{(5, 33)}$ = 14.80, p = 0.001]. Male fat IL-10 significant effect of diet [$F_{(5, 30)}$ = 4.81, p = 0.015]. Male fat IL-1β significant effect of diet [$F_{(5, 30)}$ = 3.29, p = 0.051]. n = 8 animals per group. Data are mean + SEM.

Table 4.2 Liver cytokine responses to LPS after 3 days and 3 weeks HFD

Cytokines	CL CH/Sal	SL CH/Sal	CL 3D/Sal	SL 3D/Sal	CL 3W/Sal	CL 3W/Sal	CL CH/LPS	SL CH/LPS	CL 3D/LPS	SL 3D/LPS	CL 3W/LPS	CL 3W/LPS	Main Effects
Males													
IL-1α	58.1 (10)	40.9 (6)	50.9 (5)	44.1 (5)	70.3 (11)	41.5 (6)	247.5 (88)	188.4 (79)	206.9 (60) *	190.7 (75)	186.5 (68)	116.1 (31)	LPS
IL-1β	807.7 (120.2)	543.5 (66)	682.6 (65)	614.9 (71)	896.1 (132)	626.0 (32)	1533.3 (260)	1453.8 (358)	1559.5 (282)	1487.7 (463)	1643.0 (455)	1114.6 (242)	LPS, SEX
IL-2	174.2 (23)	129.3 (17)	155.4 (14)	132.9 (10)	177.3 (13)	142.2 (6)	164.2 (6)	150.4 (22)	148.8 (13)	151.3 (34)	166.7 (21)	116.8 (15)	LITTER, SEX
IL-4	1018.5 (190)	843.0 (309)	884.5 (297)	682.0 (182)	1040.4 (336)	853.6 (195)	856.6 (232)	1031.4 (341)	721.0 (185)	1163.8 (516)	1081.3 (415)	738.1 (212)	SEX
IL-5	1205.4 (167)	966.9 (185)	1056.8 (223)	905.0 (142)	1163.2 (253)	1034.3 (163)	1007.9 (175)	1151.6 (266)	879.1 (100)	1160.0 (311)	1099.5 (247)	964.1 (181)	SEX
IL-6	84.9 (12)	57.4 (12)	72.7 (10)	56.2 (7)	88.3 (10)	68.8 (11)	106.0 (14)	102.5 (29)	94.2 (18)	109.1 (36)	103.7 (26)	71.4 (10)	LPS, SEX
IL-10	4885.6 (654)	3760.2 (896)	4331.9 (794)	3655.3 (472)	5228.0 (1058)	4326.8 (660)	4764.5 (699)	4952.8 (1105)	4140.0 (567)	5372.0 (1667) #	5021.6 (1108)	3824.8 (680)	SEX
IL-12	341.2 (59)	260.9 (64)	277.0 (84)	224.4 (55)	314.5 (94)	257.9 (61)	263.5 (61)	311.1 (84)	233.2 (49)	316.1 (112)	289.9 (95)	249.0 (61)	SEX
TNFα	205.7 (31)	157.0 (36)	173.9 (22)	158.8 (19)	221.3 (20)	168.6 (23)	287.2 (47)	232.4 (58)	243.8 (45)	257.9 (67)	284.7 (77)	194.2 (20)	LPS, SEX
Females													
IL-1α	40.7 (3)	35.6 (5)	44.1 (5)	37.7 (4)	34.5 (2)	68.0 (14)	155.3 (41)	179.0 (28)	292.0 (71)	136.2 (35)	151.8 (26)	167.4 (14)	

IL-1β	424.1 (54)	324.3 (56)	459.6 (45)	390.7 (35)	377.0 (30)	524.9 (76)	975.4 (172)	1328.1 (227)	1403.6 (241)	930.8 (157)	964.0 (166)	1084.8 (113)	
IL-2	122.1 (7)	93.5 (10)	126.1 (12)	111.6 (11)	107.2 (8)	111.6 (8)	102.9 (14)	100.4 (11)	105.3 (9)	93.5 (13)	106.3 (7)	119.1 (14)	
IL-4	406.5 (56)	313.9 (88)	586.7 (133)	414.1 (116)	369.1 (59)	465.9 (72)	366.1 (96)	287.8 (49)	271.4 (43)	405.7 (83)	350.1 (48)	498.3 (129)	
IL-5	440.0 (36)	399.3 (37)	526.6 (50)	417.1 (37)	423.0 (25)	563.7 (69)	405.4 (58)	412.3 (47)	354.7 (35)	427.3 (59)	397.2 (13)	500.8 (61)	
IL-6	56.0 (7)	41.7 (6)	55.0 (6)	46.3 (5)	42.5 (4)	51.7 (7)	62.8 (8)	68.9 (10)	81.7 (16)	55.1 (9)	55.7 (5)	74.3 (10)	
IL-10	2206.2 (116)	1798.6 (162)	2223.9 (150)	1995.1 (226)	2021.2 (165)	2009.6 (85)	1849.1 (226)	1865.4 (143)	2002.2 (176)	1891.4 (162)	2043.1 (84)	2212.5 (128)	
IL-12	69.8 (7)	50.7 (9)	80.9 (11)	65.5 (8)	61.1 (6)	88.0 (14)	60.7 (11)	67.1 (13)	52.8 (7)	65.8 (11)	60.6 (5)	82.1 (13)	
TNFα	162.2 (27)	124.4 (19)	129.2 (15)	129.0 (21)	105.1 (13)	133.0 (25)	159.4 (23)	182.2 (32)	203.0 (52)	142.0 (25)	145.9 (13)	182.8 (32)	

Table 4.2 Liver cytokine (pg/mL) in response to lipopolysaccharide (LPS) after 3 days (3D) and 3 weeks (3W) in rats that were raised in control (CL) and small (SL) litters. Interleukin (IL)-1α: significant effect of LPS [$F_{(23, 118)} = 70.78$, $p < 0.001$]. IL-1β: significant effect of LPS [$F_{(23, 117)} = 77.74$, $p < 0.001$]; significant effect of sex [$F_{(23, 117)} = 14.28$, $p < 0.001$]. IL-2: significant effect of litter size [$F_{(23, 119)} = 8.47$, $p = 0.004$]; significant effect of sex [$F_{(23, 119)} = 52.93$, $p < 0.001$]. IL-4: significant effect of sex [$F_{(23, 120)} = 34.36$, $p < 0.001$]. IL-5: significant effect of sex [$F_{(23, 120)} = 101.25$, $p < 0.001$]. IL-6: significant effect of LPS [$F_{(23, 120)} = 15.14$, $p < 0.001$]; significant effect of sex [$F_{(23, 120)} = 22.68$, $p < 0.001$]. IL-10: significant effect of sex [$F_{(23, 120)} = 93.59$, $p < 0.001$]. IL-12: significant effect of sex [$F_{(23, 120)} = 95.10$, $p < 0.001$]. Tumour necrosis factor (TNF) α: significant effect of LPS [$F_{(23, 120)} = 14.08$, $p < 0.001$]; significant effect of sex [$F_{(23, 120)} = 20.84$, $p < 0.001$]. *: Versus saline group n = 8 animals per group, $p < 0.05$. #: Versus female group n = 8 animals per group, $p < 0.05$. Data are mean +SEM.

4.3.7 Neuronal activation in brain regions involved in fever regulation and the response to LPS

Next we examined neuronal activation in several brain regions involved in fever regulation and the response to LPS. We found CL rats given 3 days HFD responded to LPS with a six-fold increase in neuronal activation in the PVN relative to chow fed and saline injected in males. As observed previously, neonatal overfeeding increased PVN neuronal activation in response to LPS under chow-fed conditions, but with 3 days HFD the SL rats were no longer responsive to LPS. 3 weeks HFD abolished LPS responses in both CL and SL groups. (Figure 4.12A and B) CL females had a similar increase relative to saline injected (Figure 4.12F and G), similar responses were also seen in the ventral bed nucleus of the stria terminalis (vBNST) (Figure 4.12C and H) and the ventromedial preoptic area (VMPOA) in males (Figure 4.12D and I), with LPS leading to increased Fos positive cells in these regions compared with saline after 3 days HFD in control but not overfed rats. Specifically, there was an increase Fos positive cells in vBNST after LPS-treatment in control males with 3 days HFD compared with saline-treated control males with 3 days HFD, but no other relevant differences. In male and female vBNST, there was an LPS, sex interaction, a litter size, sex interaction, and a litter size, diet interaction, but there were no male – female differences with *post hoc* tests. In the VMPOA there was again a significant increase in Fos positive cells in LPS-treated rats after 3 days HFD in control males compared with saline-treated controls with 3 days HFD, but there were no other relevant differences. Between male and female VMPOA, there were no differences with *post hoc* tests (Figure 4.12D and I). In the vascular organ of the lamina terminalis (OVLT) there were no relevant differences with *post hoc* tests except that in females there were more Fos-positive cells with LPS after 3 weeks HFD in controls than in overfed rats (Figure 4.12E and J).

Figure 4.12 LPS induced neuronal activation in nenatally overfed rat brain

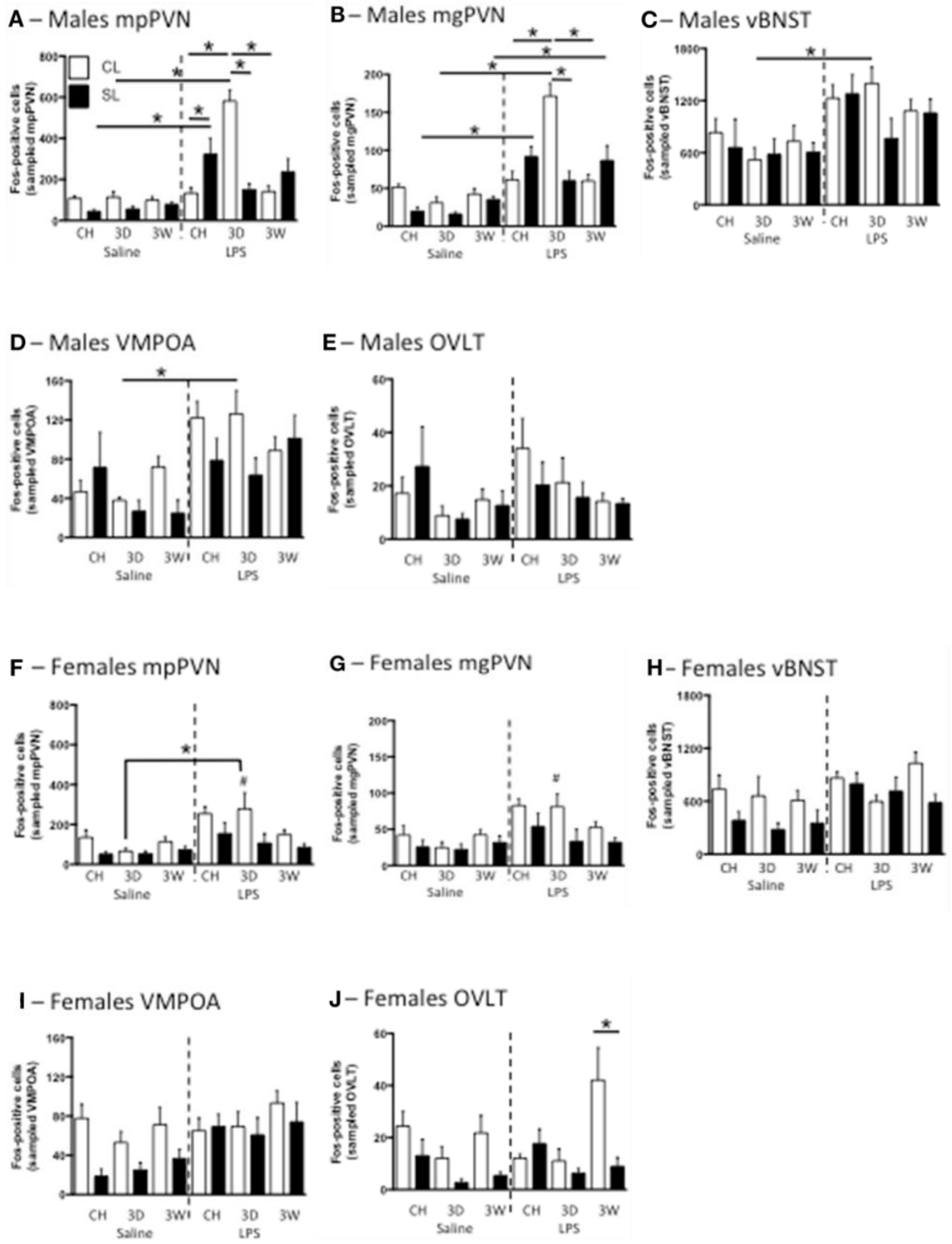


Figure 4.12 Effects of neonatal overfeeding on neuronal activation in response to LPS. Neuronal activation in the medial parvocellular (mp) (A and F) and magnocellular (mg) (B and G) paraventricular nucleus of the hypothalamus (PVN), the ventral bed nucleus of the stria terminalis (vBNST) (C and H), the ventromedial

preoptic area (VMPOA) (D and I) and the vascular organ of the lamina terminalis (OVLT) (E and J) with LPS after 3 days (3D) and 3 weeks (3W) HFD or chow (CH) in male (A – E) and female (F – J) adult rats that were raised in control (CL) and small litters (SL). mpPVN: significant effect of litter size [$F_{(23, 140)} = 15.09$, $p < 0.001$], LPS [$F_{(23, 140)} = 68.11$, $p < 0.001$], diet [$F_{(23, 140)} = 3.99$, $p = 0.02$], and sex [$F_{(23, 140)} = 7.64$, $p < 0.001$] and a significant litter size, LPS, diet, sex interaction [$F_{(23, 140)} = 5.22$, $p = 0.007$]. mgPVN: significant effect of litter size [$F_{(23, 140)} = 16.85$, $p < 0.001$], LPS [$F_{(23, 140)} = 71.26$, $p < 0.001$], and sex [$F_{(23, 140)} = 12.35$, $p = 0.001$] and a significant litter size, LPS, diet, sex interaction [$F_{(23, 140)} = 3.78$, $p = 0.025$]. vBNST: significant effect of sex [$F_{(23, 131)} = 15.76$, $p < 0.001$], LPS [$F_{(23, 131)} = 31.73$, $p = 0.051$], and litter size [$F_{(23, 131)} = 8.05$, $p = 0.005$] and a significant litter size, LPS × diet × sex interaction [$F_{(23, 131)} = 3.05$, $p = 0.051$]. VMPOA: significant effect of LPS [$F_{(23, 130)} = 31.81$, $p < 0.001$] and litter size [$F_{(23, 130)} = 11.70$, $p < 0.001$] as well as a significant litter size × LPS × diet × sex interaction [$F_{(23, 130)} = 3.91$, $p = 0.023$]. OVLT: significant effect of diet [$F_{(23, 133)} = 5.34$, $p = 0.006$] and litter size [$F_{(23, 133)} = 7.64$, $p = 0.007$]. There was also a diet × sex interaction of $p < 0.06$ [$F_{(23, 133)} = 2.90$, $p = 0.058$]. #: Sex difference between corresponding groups. *: As indicated, $p < 0.05$. n = 8 animals per group. Data are mean + SEM.

Figure 4.13 Fos stained cells in male rat PVN

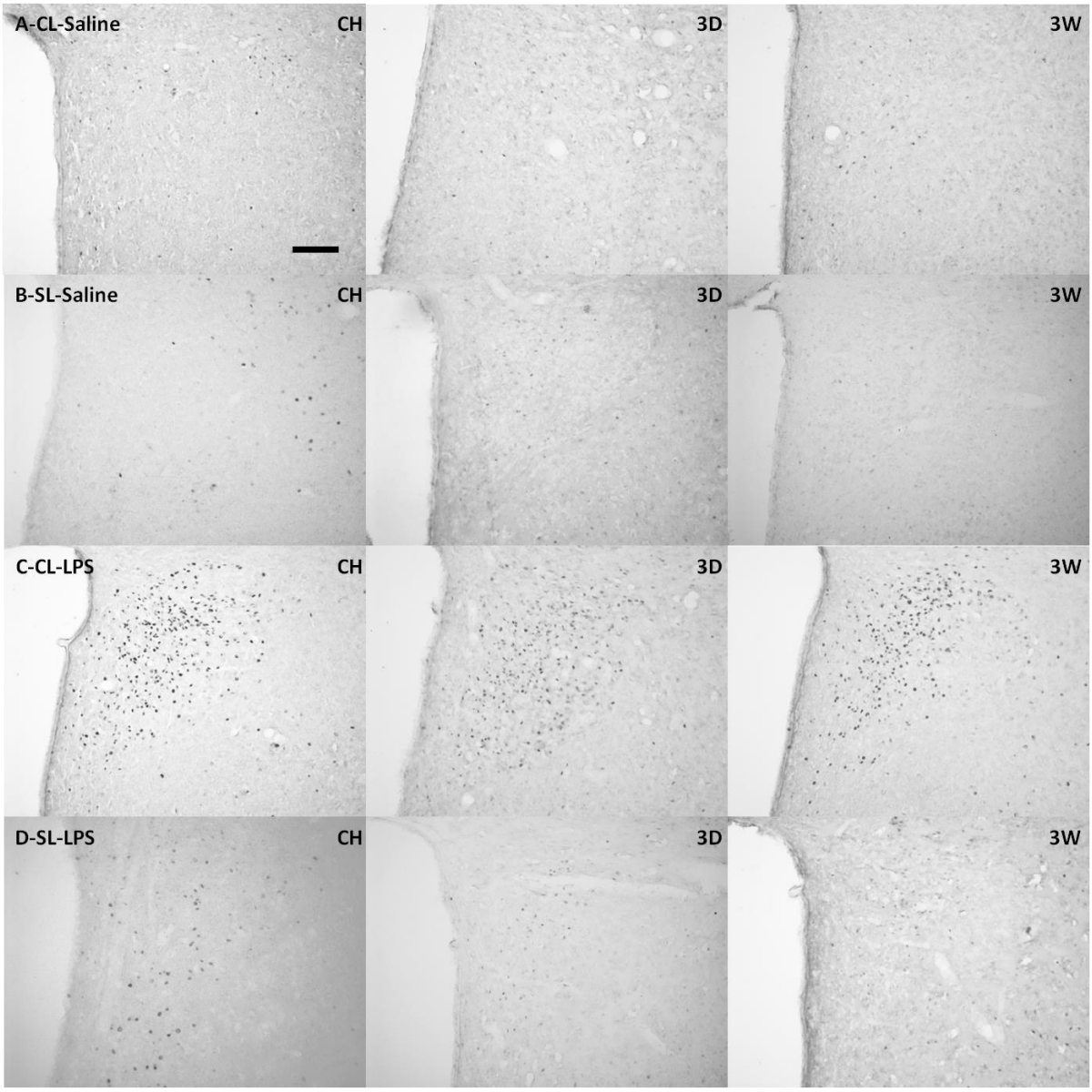


Figure 4.13 A – D) Representative photomicrographs of the PVN from males illustrating differences in numbers and density of c-Fos-stained cells.20× magnification, scale bar = 50 µm.

Figure 4.14 Fos stained cells in female rat PVN

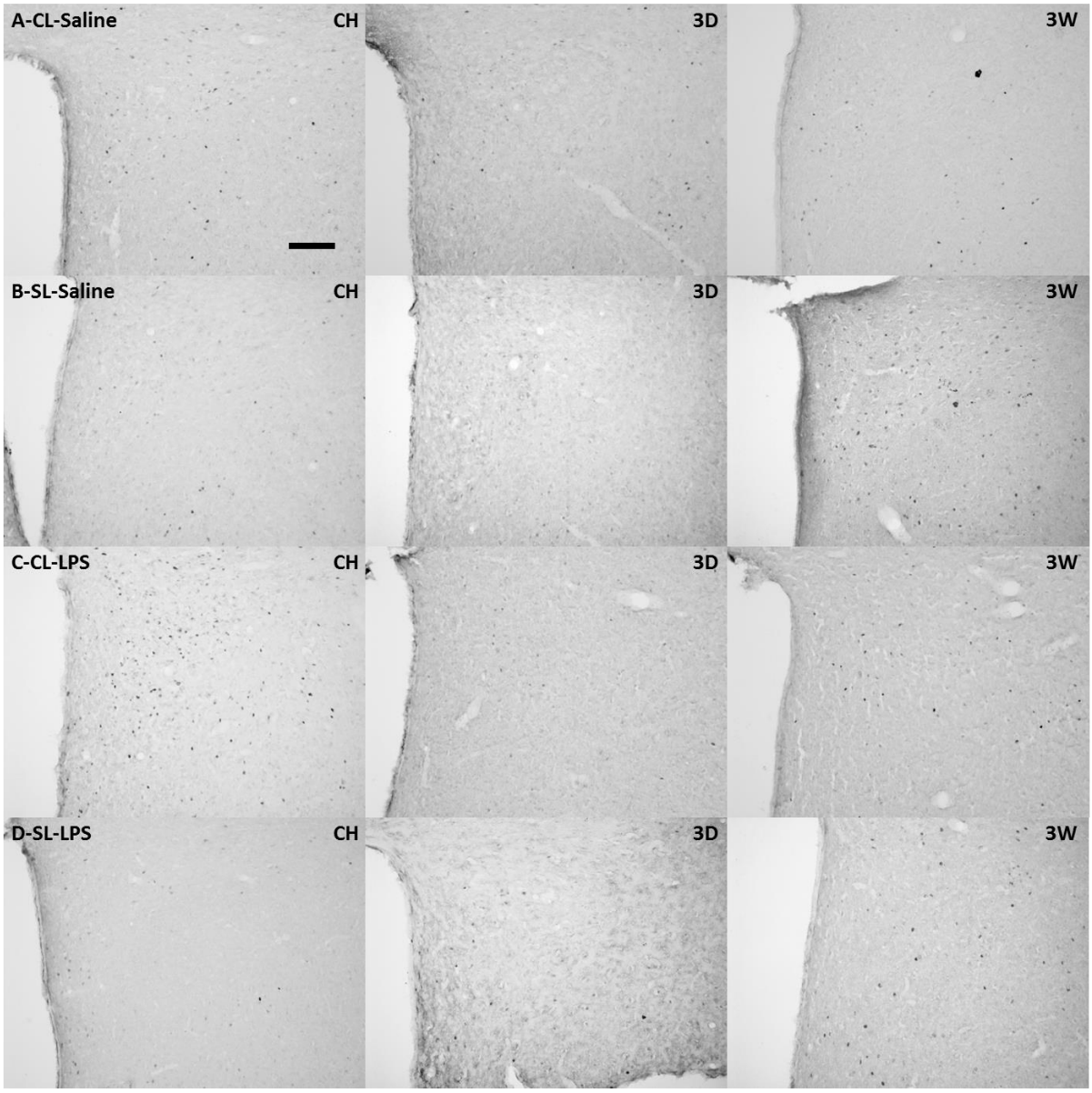


Figure 4.14 A – D) Representative photomicrographs of the PVN from females illustrating differences in numbers and density of Fos stained cells.20× magnification, scale bar = 50 µm.

4.4 Discussion

In this study, we have shown neonatal overfeeding causes body weight increases that persist into adulthood, but this has only a slight effect on the metabolic health of the animal and responses to an obesogenic diet. The neonatal overfeeding increased rat plasma and liver triglyceride levels and the high fat diet reduced energy consumption efficiency in the neonatally overfed only. However, there were no differences in inflammatory cytokines in liver or fat between the groups. The major difference the results show is if we gave male SL rats 3 days HFD, this led to a response to LPS that was six-fold lower than in male controls in their neuronal activation in PVN. We did not see differences in LPS / fever-regulatory brain regions (VMPOA, OVLT, vBNST). However, we did not directly measure the fever and sickness behaviour in these animals. We also see the responses were somewhat different in females compared with males.

In our study, we specifically chose to test high fat feeding for 3 days, given our hypothesis that neonatally overfed rats may be more sensitive to HFD than controls. As these studies showed, our neonatally overfed rats were not metabolically affected by 3 days or 3 weeks HFD, having no differences in weight, glucose tolerance, or liver triglycerides. Similarly, Maric et al.'s studies did not find any difference between chow and HFD when rats were supplied a HFD (32% kilocalories as fat) for 8 weeks. They also showed only butter based HFD was able to slightly increase the fat pad weight and the total body weight compared with chow diet in Wistar rats. There were no effects when the rats were fed a coconut oil-based diet, indicating the different kinds of saturated fat may lead to differences in the inflammatory effects (Maric, Woodside et al. 2014). They also showed the inflammatory effects of HFD in the rat are assisted by food-novelty-related increases in glucocorticoids (GCs) (Maric, Woodside et al. 2014). It is possible different types of

saturated fat could have different effects on our model. As previous studies have shown, lard (long-chain saturated fatty acids) and olive oil (monounsaturated fatty acids) can induce classical HFD effects, fish oil and medium-chain saturated fatty acid-based HFD did not induce insulin resistance, long-chain saturated fatty acid and monounsaturated fatty acids can cause hepatic steatosis (Buettner, Parhofer et al. 2006). Thus, we would expect that if we gave neonatally overfed animals different oil based HFD, the effects of neonatal overfeeding would be exacerbated by long-chain saturated fatty acids and monounsaturated fatty acids, but would be prevented by the polyunsaturated fatty acids (fish oil) and medium-chain fatty acids. This would be an interesting avenue for future studies.

One of the most interesting findings from our results was to show that central pro-inflammatory changes can occur in the absence of significant metabolic or peripheral pro-inflammatory profile changes. We showed that neonatal overfeeding significantly increased male PVN microglial numbers, SL males had more microglia than CL. 3 days HFD caused a substantial increase in microglial numbers and density in CL males, but caused a reduction in microglial numbers in SL. Microglial numbers remained elevated in CL males. The 3 weeks HFD increased microglial density in CL males but not SL, and the 3 week HFD had little effect on SL males. In females, the responses were more ambiguous, with neonatal overfeeding and adult HFD having no significant effects (Cai, Dinan et al. 2014). From global inflammatory responses studies, we found the liver TLR4 gene expression was increased in both controls and neonatally overfed groups by 3 days HFD. However, we could not see any significant effects on peripheral obesity indicators or tissue inflammatory cytokines at this time point. Similar findings have been published by Thaler and Maric (Thaler, Yi et al. 2012, Maric, Woodside et al. 2014). From very early studies, it was considered obesity was directly associated with the appearance of peripheral inflammation, with the levels of pro-inflammatory cytokines in circulation increasing as the obesity

increased (Hotamisligil, Arner et al. 1995, Hotamisligil 2006). Thus, the comprehensive inflammatory response to HFD and weight increase was thought to be primarily due to the adipose tissue macrophage infiltration and peripheral overproduction of pro-inflammatory cytokines. It now seems this occurs more slowly than central inflammation (Weisberg, McCann et al. 2003, Xu, Barnes et al. 2003). Our recent evidence supports data that the central inflammatory reaction and the neuronal injury induced by HFD actually precede peripheral inflammation. Thaler and his colleagues have shown that some markers of inflammatory cytokines such as IL-6, suppressor of cytokine signalling 3 (SOCS3), and inhibitor of nuclear factor κ-B kinase subunit β (IKKβ) in the hypothalamus are significantly increased after the animal had been supplied with HFD for as little as 24 hours. The 1 week HFD was sufficient to cause neuronal injury. In this study, the peripheral inflammatory cytokines were not altered until weeks to months on the HFD (Thaler, Yi et al. 2012). Other studies indicate peripheral inflammation may be delayed longer than 8 weeks after commencing HFD (Maric, Woodside et al. 2014). More information from Gao et al.'s study has also shown central inflammation in the absence of metabolic and peripheral pro-inflammatory change. In this study, the HFD effects were directly influenced by leptin treatment, which changed the central and global signalling of inflammation (Gao, Ottaway et al. 2014).

In the present study, we have also found that the short term HFD influences how the rats respond to a bacterial mimetic. Control rats that were given 3 days HFD had a response to LPS that was six-fold higher than that of the chow controls in terms of neuronal activation of the PVN. The amplitude of these responses of the PVN implies the animals would have more sickness after the LPS injection (Tarr, Chen et al. 2012). The appearances of these responses in the PVN may indicate the male rats had more of a response after the LPS stress. Interestingly, this exacerbated response was not seen in the neonatally overfed, potentially

indicating less vulnerability to sickness in this group. Moreover, our previous studies have shown that the GC release was unusually slowed in SL males (Clarke, Stefanidis et al. 2012), potentially indicating the SL are protected from central inflammation.

We did not find any differences in fever-regulatory brain areas activated by LPS in male OVLT, but we found a significant difference between CL and SL females in the number of Fos-positive nuclei in the OVLT in response to LPS after 3 weeks of high-fat diet. This may indicate that the immune responses to LPS are different between males and females after 3 weeks HFD, with the SL females havingd a markedly reduced response to LPS in the OVLT. When cytokxines are released into circulation, cyclooxygenase-2, the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin E2 (PGE2), is stimulated. PGE2 acts in the brain, principally in the OVLT and in the ventromedial preoptic area of the anterior hypothalamus, to stimulate heat conservation via cutaneous vasoconstriction and reduction of sweating or panting, and heat production via increase in brown adipose tissue metabolism (Morrison, Nakamura et al. 2008), the end result of which fever (Spencer, Galic et al. 2011). In the PVN of the hypothalamus, deoxycorticosterone-pretreatment reduces angiotensin II-induced c-Fos induction in the PVN, but increases c-Fos expression in the OVLT (Grafe, Takacs et al. 2014). These data suggest the febrile response after 3 weeks HFD would be strong in control females, but suppressed in neonatally overfed females, potentially suggesting a compromised ability to combat the immune challenge. However, we did not see the female rats had any different responses to LPS in PVN. Thus, the connection between OVLT and PVN in this study is not clear and will need further investigation. Also, analysing the fever or sickness behaviors and the corticosterone levels would be interesting elements for future work.

Previously, studies have also shown microglial reactions to LPS can depend on the

background of the animals. An early life immune challenge can significantly increase microglial activation and microglial responses to a later similar immune challenge (Bland, Beckley et al. 2010, Williamson, Sholar et al. 2011). Our group has also found similar results. The neonatal overfeeding can increase the responses to a LPS challenge in terms of microglial activation, fever, cytokines, and HPA axis responses (Clarke, Stefanidis et al. 2012, Ziko, De Luca et al. 2014). In an extension to the present study, Ilvana Ziko examined microglial responses to neonatal overfeeding and HFD (Cai, Dinan et al. 2014). In this we found 3 days HFD increases microglial numbers and density in the PVN of control rats but not the neonatally overfed. Thus, the response of the PVN to an immune challenge may be significantly affected by the interaction between primed microglia and HFD.

We also found the animals' responses to HFD are sex-dependent, particularly the responses to immune challenge between male and female animals are different. Thus, the males are more likely to be affected by 3 days HFD with an increase in microglial activation in PVN (Cai, Dinan et al. 2014) and PVN Fos responses to LPS, but there was no effect on females. We did not directly monitor female cycle stage in our experiment, because it may give females additional stress (Lovick 2012). However, it is unlikely cycle stage affected our results because the variability of the data is similar between males and females. There are very few studies in the literature reporting data from both males and females in this area of research. Senthil-Kumar provided a HFD short-term either of saturated or unsaturated fat and found male mice developed insulin resistance, but females kept insulin sensitivity (Senthil Kumar, Shen et al. 2014). Also, the male rats had insulin resistance and hypertension, but females did not when given high fructose or sucrose feeding (Galipeau, Verma et al. 2002). Thus, female rats may be less sensitive than males to the negative effects of short-term HFD.

Based on the results from above, we conclude that although the neonatal

overfeeding caused the animals to gain body weight faster, there was only a slight effect on the metabolic health and the responses to an obesogenic diet. There were no differences in inflammatory cytokines in liver or fat. However, the neonatal overfeeding attenuates acute central pro-inflammatory effects of short-term HFD. In additional, in males, the neonatal overfeeding increases PVN neuronal activation in response to LPS excitation, but this does not occur in females. These data indicate the responses to obesogenic diet are somewhat sex-dependent. The precise identification of the mechanisms behind these changes remains to be discovered.

Chapter 5

Effects of Neonatal Overfeeding on LPS-induced Responses of Catecholamine Cells in Brainstem

5.1 Introduction

In addition to the hypothalamic-pituitary-adrenal (HPA) axis, the sympatho-adrenal-medullary (SAM) axis is also critical to the stress response (Jayasinghe, Lambert et al. 2016). When animals respond to physiological stress, the HPA axis will secrete glucocorticoids (GCs) as described in Chapter 1; at the same time, the SAM axis is also activated and this leads to the release of catecholamines, adrenaline and noradrenaline, activating the cardiovascular system to cope with the changes in homeostasis (Dalin, Magnusson et al. 1993, Jayasinghe, Lambert et al. 2016). Temporally, the SAM axis is considered a more acute response stress to release the catecholamines, occurring within seconds, while the HPA axis response to stress occurs from minutes to hours (Herman and Cullinan 1997, Smith and Vale 2006, Gotlib, Joormann et al. 2008, Gaete 2016). The HPA axis and SAM axis both respond to signals from the hypothalamus, and these are dependent on hormonal feedback from the circulation (Bitsika, Sharpley et al. 2014). For example, the sympathetic branch of the autonomic nervous system will be quickly activated by the SAM axis and have effects on the body including heart, arteries, and skin. Thus, the catecholamines produced in the adrenal medulla can stimulate pupil enlargement, palm and feet sweating, also increases in heart rate and oxygen partial pressure and blood flow (Smeets 2010, Bitsika, Sharpley et al. 2014). Thus, it is important we also consider the contribution of the SAM axis to the stress response in our neonatally overfed rats.

Neonatal overfeeding can increase tyrosine hydroxylase (TH) in the left adrenal gland (Conceicao, Moura et al. 2013). TH is the enzyme that catalyses the conversion of amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (Kaufman 1995, Nagatsu 1995), the precursor of the catecholamines adrenaline and noradrenaline (Nagatsu 1995). Since TH is important for catecholamine synthesis and controls it under basal conditions in

rats, neonatally overfed animals with more TH are likely to have higher adrenal catecholamine levels (Conceicao, Moura et al. 2013). Adrenal catecholamine plays an important role in body energy consumption (Nonogaki 2000). It also helps the liver to metabolise lipid and glucose (Conceicao, Moura et al. 2013). Adrenal catecholamines can also contribute to glycogen consumption (Yorek, Rufo et al. 1980), and can increase hormone-sensitive lipase, activated in adipocytes and hydrolyses triglycerides (Djouder, Tuerk et al. 2010).

Catecholamines are also important centrally. The paraventricular nucleus of hypothalamus (PVN) receives direct catecholaminergic projections from the nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM) to influence activation of the HPA axis (Shafton, Ryan et al. 1998, Toth, Gallatz et al. 1999, Pyner and Coote 2000, Hardy 2001). The catecholamine cells in NTS and VLM play an important role in regulating responses to stress and as such many studies have shown physical and psychological stress activate catecholamine cells in these NTS and VLM regions (Ceccatelli, Villar et al. 1989, Senba, Matsunaga et al. 1993, Ericsson, Kovacs et al. 1994, Sawchenko, Li et al. 2000).

Lipopolysaccharide (LPS) was used to excite the neonatal rat's airway can significantly increase NTS pro-inflammatory cytokine concentrations interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)α within a few hours (Perenboom, Beckers et al. 1996). Similarly, an i.p. injection of LPS to adult male rats significantly increased numbers of c-Fos positive cells in NTS, as well as plasma corticosterone levels (Reyes, Abarzua et al. 2012), indicating the NTS is at least involved in the response to LPS.

As our previous work showed neonatal overfeeding exacerbates PVN responses to LPS, we hypothesised the catecholamine cell response to LPS in the brainstem would also be increased by neonatal overfeeding. Because these brainstem regions are also key modulators

of diet and feeding (Browning and Travagli 2014), we also examined their responses to LPS in the context of acute and long-term high fat diet (HFD), reasoning that neonatal overfeeding may lead to vulnerable brainstem responses to the HFD. We used brainstems that were collected from the "central pro-inflammatory effects of HFD" studies (see Chapter 4), and we analysed the catecholamine cells in NTS and VLM regions of the brainstem after stress with LPS.

5.2 Methods and materials

The methods and materials are as described in previous chapters, except where is noted below. All experiments were initiated between 0900 and 1200 hour to limit potential effects of circadian rhythms on any parameters measured. Our results in Chapter 4 showed the males are more likely to be affected by short-term HFD but there was no effect on females. Thus, the males Fos / TH activity in the brainstem becomes the priority experimental objective. In order to study the effects of the short-term and long-term HFD on neonatally overfed rat, the brainstem tissue was collected from the animals we presented in Chapter 4. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals. All these were approved by the RMIT University Animal Ethics Committee (see Appendix 3).

5.2.1 Brain tissue preparation and Fos / TH immunohistochemistry

Brain tissues were prepared for immunohistochemistry as detailed in Chapter 2. The brainstem samples were cut into 50 µm coronal sections using a cryostat. Neuronal activation was assessed in the fixed brains by quantifying Fos / TH-positive-immunoreactivity. Thus, a one-in-five series of brainstem sections from each animal was incubated in primary rabbit polyclonal Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 ^{o}C, at 1:10,000. Then the brainstem sections were incubated in secondary biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) 1:200, for 2 hours, at room temperature. We then transferred the brainstem sections into an avidin-biotin horseradish peroxidase (HRP) complex A and B (Vector Elite kit, Vector) for 1 hour, at room temperature. At this point, the brainstem sections were incubated in nickel / cobalt diaminobenzidine for 10 minutes at room temperature to visualise the HRP activity, seen as a

dark brown nuclear deposit. The reaction was terminated once there was an optimal contrast between specific cellular and non-specific background labelling. To dual-label for TH, we repeated these steps using an anti-TH antibody (Leica Biosystems, Newcastle, United Kingdom) overnight at 4 ^{o}C, at 1:200. Then the brainstem sections were incubated in secondary biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) 1:500, for 2 hours, at room temperature. We then transferred the brainstem sections into an avidin-biotin HRP complex A and B (Vector Elite kit, Vector) for 1 hour, at room temperature and omitting the nickel / colbalt, so that the cytoplasmic labelling appeared as an amber colour. The coloured brainstem sections from each treatment group were processed simultaneously, and mounted on poly-L-lysine-coated slides, dehydrated in a series of alcohols, cleared in Histoclear and cover slipped. An experimenter blinded to the group treatments performed cell counts of Fos / TH -positive nuclei in NTS and VLM (Figure 5.1). Cell counts were made from 11 in sections for each animal, 5 rostrally and 5 caudally to obex.

Figure 5.1 Fos / TH positive cells in NTS and VLM of brainstem section

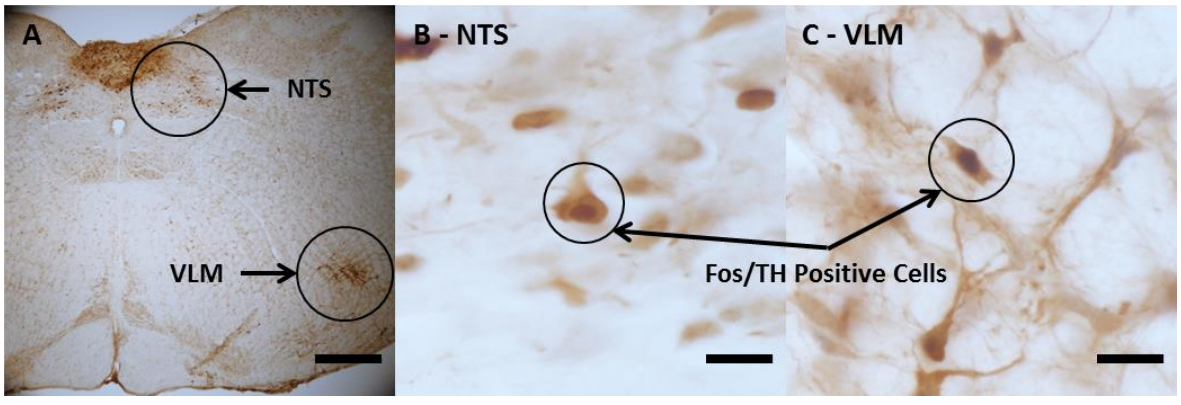


Figure 5.1 A) Activated TH cells in brainstem; 4×magnification, scale bar = 500 µm. B) Nucleus tractus solitarius (NTS); 80× magnification, scale bar = 25 µm C) Ventrolateral medulla (VLM): 80× magnification, scale bar = 25 µm.

5.2.2 Data analysis

In order to count the reactions from different parts of each brainstem region, we binned the regions into three bins (most caudal, middle and most rostral) of 3 sections, using obex as a marker. We calculated the total activated Fos / TH cells in these 3 sections of brainstem. To compare basal results between CL and SL rats, an analysis of variance (ANOVA) with repeated measures was used, with rostral-caudal level as the repeated measure. When a significant interaction was found between litter size and treatments, Tukey's *post hoc* were performed. Data are presented as the mean + standard error of the mean (SEM). Statistical significance was assumed when $p < 0.05$.

5.3 Results

5.3.1 Effects of neonatal overfeeding on LPS-induced responses of catecholamine cells in the nucleus tractus solitarius (NTS)

Neonatal overfeeding did not affect the number of Fos-positive TH cells in the NTS under basal conditions (Figure 5.2A). Most of the activated TH neurons were found caudal to or around obex (Figure 5.2B – G). Surprisingly, LPS did not significantly increase the number of activated TH cells in any of the groups (Figure 5.2E, F and G), although in the same rats this dose was sufficient to activate PVN (see Chapter 4). When we binned the rostrocaudal regions into three bins of 3 sections, short- (3 days) and long- (3 weeks) term HFD did not significantly change the number of activated TH cells from caudal to or around obex in any of the groups (Figure 5.2H and I). However, compared to CL the neonatally overfed rats' caudal NTS TH cell activation was lower after LPS injection in the chow-fed (Figure 5.2H). Moreover, the LPS caused a significant reduction in numbers of Fos-positive TH cells in SL that were fed 3 days HFD (Figure 5.2H). Although, LPS did not significantly increase the activation of TH neurons in the rostral NTS in the chow or 3 weeks HFD groups, we see control and neonatally overfed rats respond to an LPS injection with increased activation of TH cells if they had been fed 3 days HFD compared with all non-stressed groups (Figure 5.2J). In addition, activation was increased in the 3 days HFD group post-LPS relative to chow if the rats had been overfed as neonates.

Figure 5.2 Neuronal activation of NTS in male rats

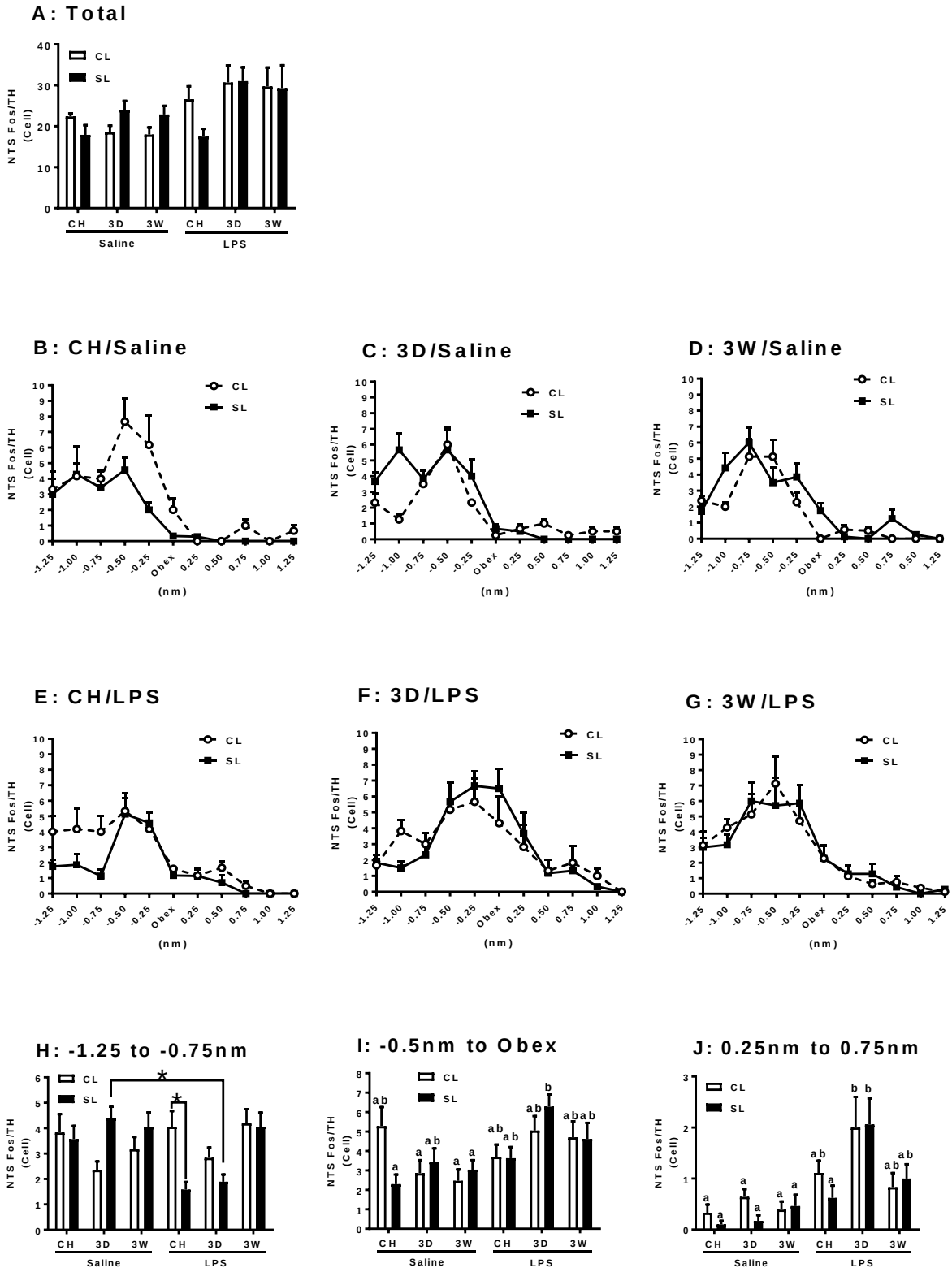


Figure 5.2 A) The total activated TH cells in nucleus tractus solitarius (NTS) of brainstem; significant LPS [$F_{(2, 901)} = 8.263$ $p = 0.004$], litter × HFD [$F_{(2, 901)} = 4.353$ $p = 0.013$], and LPS × HFD [$F_{(2, 901)} = 23.117$ $p = 0.045$]. B

– G) The Fos / TH positive cells in NTS, in control (CL) and neonatally overfed (SL) adult males, that were supplied with chow (CH), 3 days (3D), and 3 weeks (3W) high fat diet (HFD) after saline or LPS stress. H – J) The binned rostrocaudal regions (three bins of 3 sections). H: Significant HFD [$F_{(2, 237)} = 4.101$ $p = 0.018$], litter × LPS [$F_{(1, 237)} = 12.471$ $p < 0.001$], litter × HFD [$F_{(2, 237)} = 4.205$ $p = 0.016$], and LPS × HFD [$F_{(2, 237)} = 2.97$ $p = 0.053$]. I: Significant LPS [$F_{(1, 237)} = 12.966$ $p < 0.001$], litter × HFD [$F_{(2, 237)} = 3.157$ $p = 0.044$], and LPS × HFD [$F_{(2, 237)} = 3.851$ $p = 0.023$]. J: Significant LPS [$F_{(1, 237)} = 31.416$ $p < 0.001$], HFD [$F_{(2, 237)} = 6.046$ $p = 0.003$], and LPS × HFD [$F_{(2, 237)} = 4.426$ $p = 0.013$]. “a and b” different letters indicate significant differences $p < 0.05$. *: $p < 0.05$. n value for saline is CL/CH: 6, CL/3D: 6, CL/3W: 8, SL/CH: 7, SL/3D: 7, SL/3W: 8 animals; LPS is CL/CH: 6, CL/3D: 6, CL/3W: 8, SL/CH: 7, SL/3D: 7, SL/3W: 7 animals. All data are mean + SEM.

Figure 5.3 Fos / TH positive cells in NTS

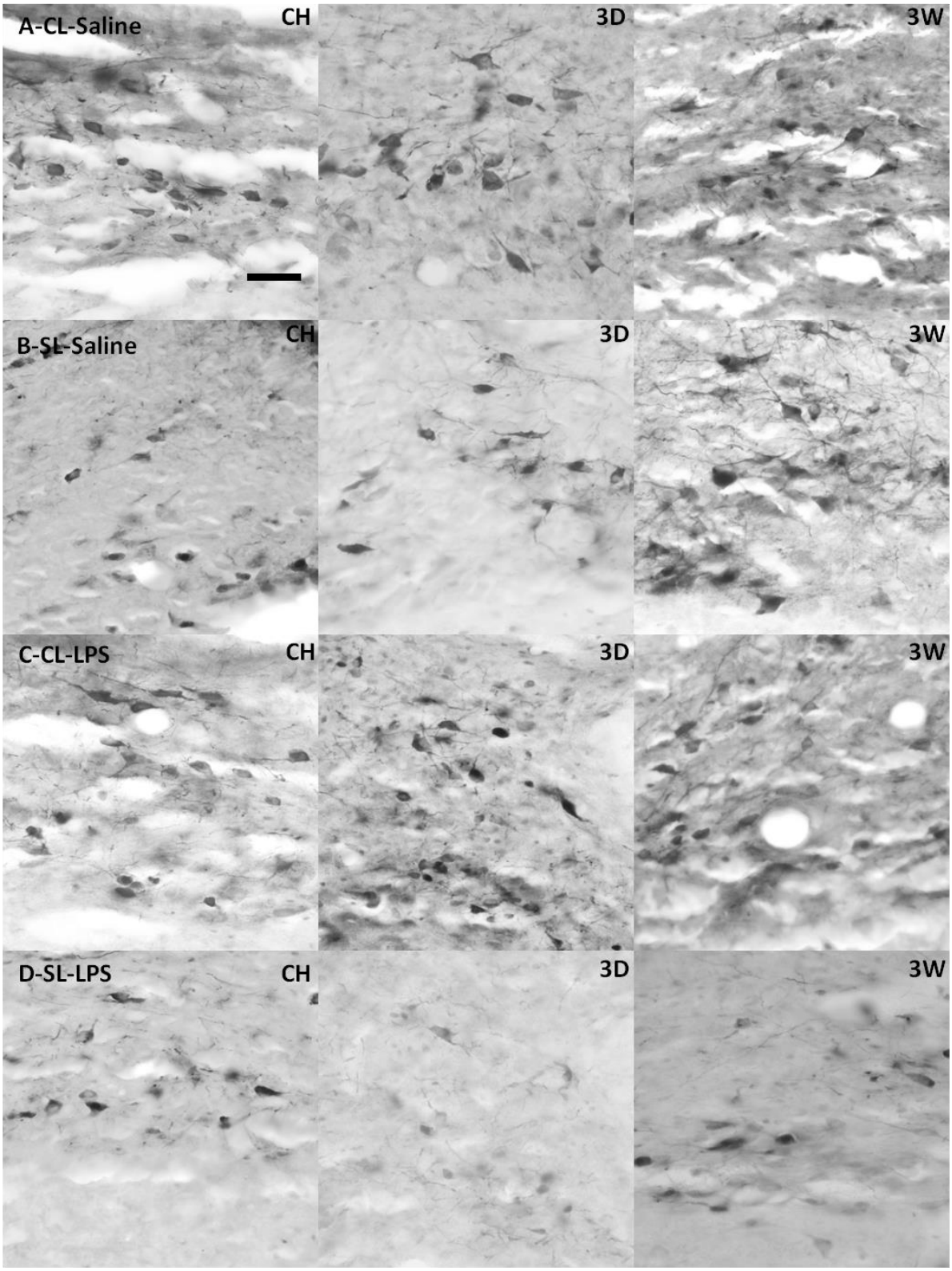


Figure 5.3 A – D) Fos / TH positive cells in nucleus tractus solitarius (NTS): 40× magnification, scale bar = 50 µm.

5.3.2 Effects of neonatal overfeeding on LPS-induced responses of catecholamine cells in the ventrolateral medulla (VLM)

Total numbers of activated TH cells in VLM were significantly increased after LPS injection in both the control and neonatally overfed groups. However, the effects of HFD on CL and SL were similar, even after LPS challenge. In animals with chow diet, LPS activated significantly more neurons in VLM of SL than CL (Figure 5.4A) Most of the activated TH neurons in the VLM were found evenly spread throughout the VLM. (Figure 5.4B – G)

When we binned the rostrocaudal regions into three bins of three sections, we found control rats fed with 3 weeks HFD had increased activation of caudal TH neurons by LPS compared with the controls fed with chow and 3 days HFD, while the neonatally overfed rats did not. There were no significant differences between CL and SL when just i.p.-injected with saline. (Figure 5.4H).

Mid VLM neuronal activation was increased with LPS compared with saline treatment in both control and neonatally overfed groups fed 3 weeks HFD (Figure 5.4I). In the rostral region, LPS significantly increased the number of activated TH cells in both short- and long- term HFD in both control and neonatally overfed groups. There were no significant differences between CL and SL when just i.p.-injected with saline. (Figure 5.4J)

Figure 5.4 Neuronal activation of VLM in male rats

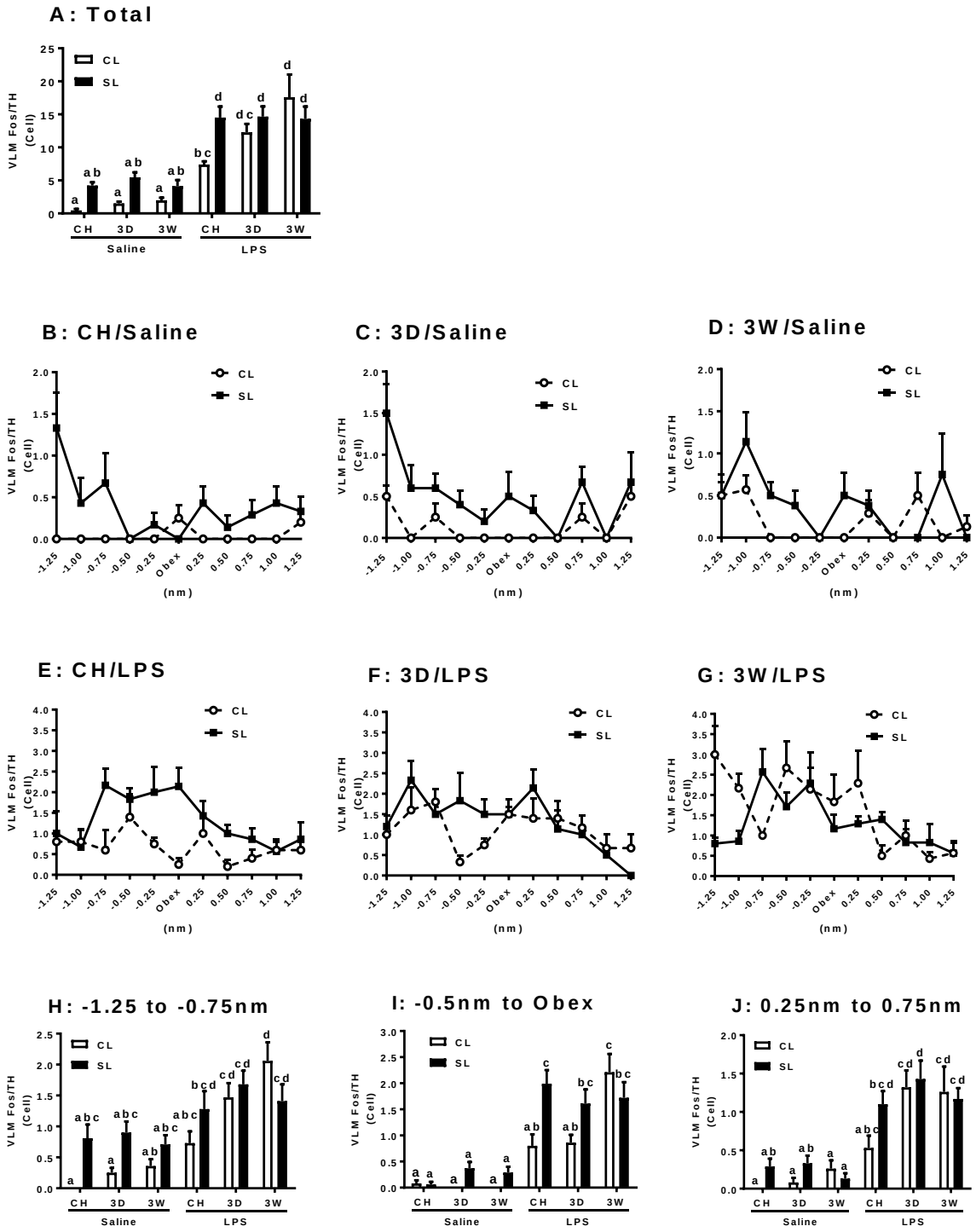


Figure 5.4 A) The total activated TH cells in ventrolateral medulla (VLM) of brainstem; significant litter size [$F_{(1, 901)}$ = 16.302 p < 0.001], LPS [$F_{(1, 901)}$ = 205.586 p < 0.001], HFD [$F_{(2, 901)}$ = 6.433 p = 0.002], litter × HFD [$F_{(2, 901)}$ = 7.235 p = 0.001], LPS × HFD [$F_{(2, 901)}$ = 3.76 p = 0.024], and litter × LPS × HFD [$F_{(2, 901)}$ = 3.757 p = 0.024]. B – G) The Fos / TH positive cells in VLM, in control (CL) and neonatally overfed (SL) adult males, that were supplied with chow (CH), 3 days (3D), and 3 weeks (3W) HFD after saline or LPS stress. H – J) The binned rostrocaudal region (three bins of 3 sections). H: Significant litter size [$F_{(1, 237)}$ = 6.879 p = 0.009], LPS

[$F_{(1, 237)}$ = 53.392 p < 0.001], HFD [$F_{(2, 237)}$ = 4.74 p = 0.01], litter × LPS [$F_{(1, 237)}$ = 5.405 p = 0.021], and litter × HFD [$F_{(2, 237)}$ = 4.16 p = 0.017]. I: Significant litter size [$F_{(1, 237)}$ = 8.841 p = 0.003], LPS [$F_{(1, 237)}$ = 144.104 p < 0.001], HFD [$F_{(2, 237)}$ = 3.923 p = 0.021], litter × HFD [$F_{(2, 237)}$ = 3.897 p = 0.022], LPS × HFD [$F_{(2, 237)}$ = 3.906 p = 0.021], and litter × LPS × HFD [$F_{(2, 237)}$ = 6.453 p = 0.002]. J: Significant LPS [$F_{(1, 237)}$ = 89.489 p < 0.001] and HFD [$F_{(2, 237)}$ = 3.27 p = 0.04]. “a, b, c, and d” different letters represent statistical differences. Columns marked with the same letter (or no letter) indicate they are not statistically significantly different from each other with Tukey’s post hoc comparisons; p < 0.05. n value for saline is CL/CH: 6, CL/3D: 6, CL/3W: 8, SL/CH: 7, SL/3D: 7, SL/3W: 8 animals; LPS is CL/CH: 6, CL/3D: 6, CL/3W: 8, SL/CH: 7, SL/3D: 7, SL/3W: 7 animals. All data are mean + SEM.

Figure 5.5 Fos / TH positive cells in VLM

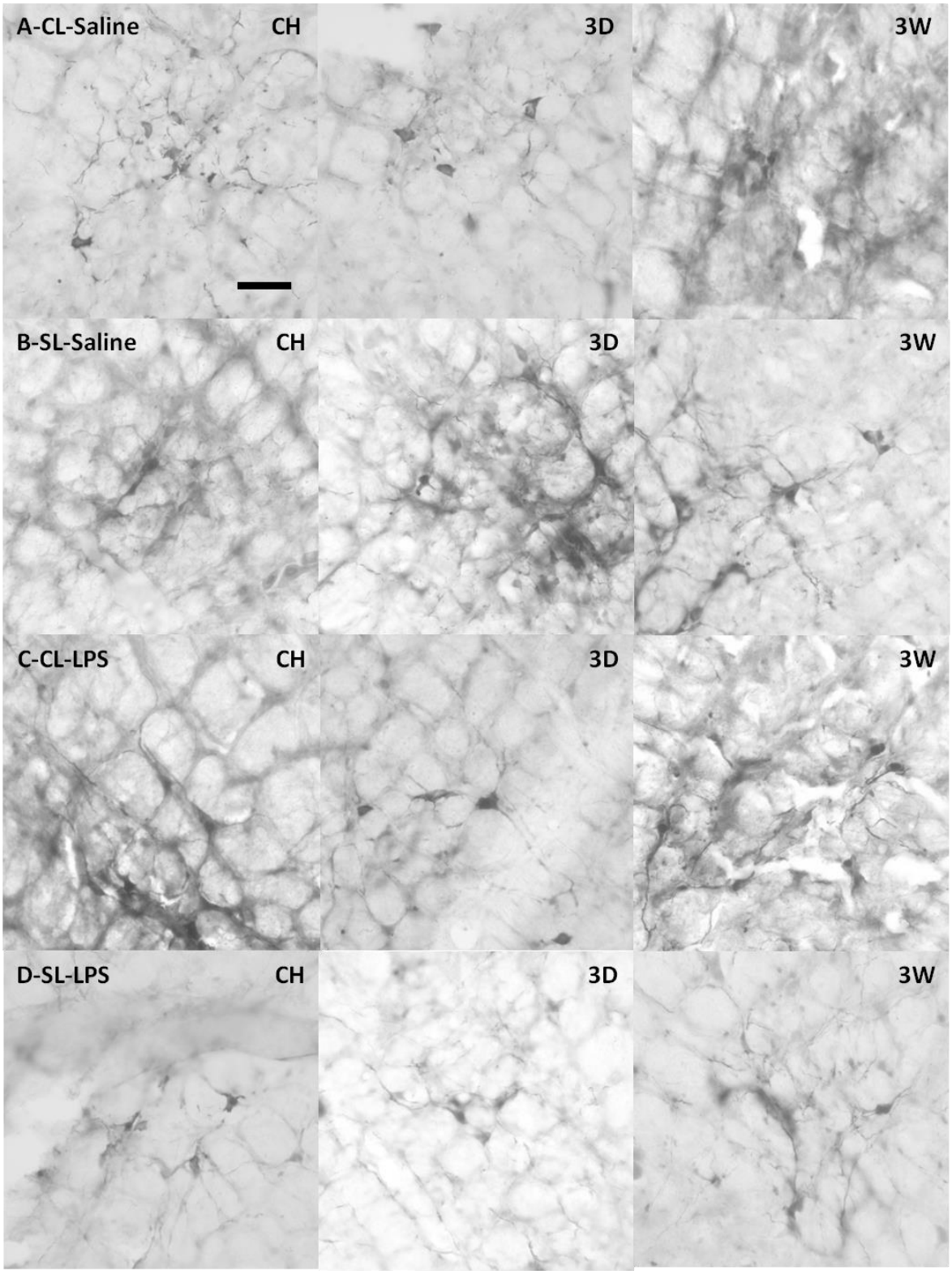


Figure 5.5 A – D) Fos / TH positive cells in ventrolateral medulla (VLM): 40× magnification, scale bar = 50 µm.

5.4 Discussion

In the NTS, under chow-fed conditions, LPS did not increase activation of TH cells. However the number of activated TH positive cells in most caudal, middle and most rostral NTS was altered under certain dietary conditions after exposure to the immune challenge. Conversely, in the VLM, LPS significantly exacerbated the activation of TH cells in all of the groups. Moreover, 3 weeks HFD significantly increased the response to LPS in control rats, but did not lead to differences in neonatally overfed rats after LPS. These findings indicate that long-term HFD increases inflammatory responses in VLM in normal rats. These data also indicate that neonatal overfeeding modifies the NTS and VLM responses to LPS under certain dietary conditions.

Our findings are somewhat different from previously published literature. An i.p. injection of LPS to adult male rats significantly activates cells in NTS (Reyes, Abarzua et al. 2012), whereas in our study we saw only a main effect of LPS with no significant *post hoc* effects of LPS under chow-fed conditions. This may reflect the fact that Reyes's work did not assess the cell phenotype, reporting total activated cells in the NTS. Godino and colleagues used i.v. isotonic blood volume expansion to stress adult male rats and the Fos / TH cell numbers were significantly increased in the NTS and caudal VLM, but not rostral VLM (Godino, Giusti-Paiva et al. 2005). However, our VLM results show LPS-activated TH neurons were evenly spread throughout the VLM. This last result is possibly due to different types of stress activating different rostrocaudal populations of the VLM. For instance, in Dayas and his colleagues' studies, they used haemorrhage, IL-1β, or restraint, noise, and forced swim to stress animals and numbers of Fos positive TH cells were increased in rostral and caudal parts of the NTS and VLM differently depending on stress type; the physical stressors, IL-1β and haemorrhage, activating rostral and the psychological stressors, restraint,

noise and forced swim, activating caudal NTS and VLM (Dayas, Buller et al. 2001, Dayas, Buller et al. 2001, Dayas, Buller et al. 2004). Furthermore, the responses to LPS in the brainstem may depend on the LPS dosage, for example, the dosage and the LPS serotype is different between Xu's experiment, where they saw LPS-induced NTS activation, and ours (Xu, Guo et al. 2003). Although our LPS effect, particularly in the NTS, was small, we did see a significant main effect of LPS that suggests it was effective.

Notably, the different rostrocaudal aspects of the NTS and VLM also have different roles in the stress response. The caudal NTS neurons are the major fibers controlling cardiorespiratory function (Zoccal, Furuya et al. 2014). Our studies showed the SL males, which were fed the chow diet, had significantly less Fos-positive TH cells in the caudal NTS in response to LPS compared with the CL males, this may suggest that the neonatal overfeeding could lead to these animals having long-term problems with cardiovascular function. There were also differences in the patterns of activation of the neurons of the NTS and VLM in response to LPS, this may be related to the fact that the PVN projects to NTS and VLM through different pathways. The NTS and VLM also cross-project and control each other. NTS projects to VLM to relay sympatho-inhibitory reflexes (Cheyuo, Jacob et al. 2012), and VLM projects to NTS (Dormer, Anwar et al. 1993). Previous studies have shown of the neurons activated by stimulation of the central nucleus of the amygdala, 20% in the VLM and 3% in the NTS contained retrograde tracer and were also immunopositive for tyrosine hydroxylase, the enzyme responsible for synthesis of catecholamines (Petrov, Jhamandas et al. 1996).

The NTS receives excitatory afferent vagal input (Paton, Li et al. 2000) and can inhibit the sympathetic output through a direct excitatory glutaminergic input to the caudal VLM (Aicher, Kurucz et al. 1995). The NTS also acts via excitatory glutaminergic projection

to the nucleus ambiguus, to inhibit rostral VLM sympathetic discharge (Agarwal and Calaresu 1992). In addition, NTS directly drives the chemoreceptive regions of VLM during peripheral chemoreceptor activation (Sun and Reis 1994). The function of the PVN drives to the VLM is to increase the sympathetic efferent output, and these are more related to the regulation of the arterial pressure (Rossi and Chen 2001, Tjen, Guo et al. 2016). However, the PVN→VLM activation may be responsible for driving the autonomic responses to LPS. If this is true, then we may expect the physiological response to LPS in chow fed CL animals to be different from the chow fed SL animals in terms of their output from the PVN to VLM.

In this study we hypothesized that neonatal overfeeding would exacerbate brainstem catecholamine responses to LPS. Previous studies have shown neonatal overfeeding increased TH in the left adrenal gland (Conceicao, Moura et al. 2013), and we had previously seen neonatal overfeeding exacerbates HPA axis responses to LPS. Although the number of TH positive cells in the NTS after LPS was not affected, the responses of the VLM were increased in the neonatally overfed relative to controls. The PVN receives direct catecholaminergic projections from the NTS and VLM to influence activation of the HPA axis (Buller 2010). However, NTS is known to relay visceral information to the PVN though noradrenaline, whereas VLM is known to relay visceral information to the PVN though adrenaline (Smith and Vale 2006). Previous studies have shown blocking the inputs from the VLM to the PVN by cyclooxygenase inhibitors can decrease PVN neuronal activation in response to stress and LPS (Xu, Guo et al. 2003). It is therefore possible that neonatal overfeeding is able to modify the VLM inputs to the PVN, accounting for the exacerbated stress responses we see, additional to changes in melanocortin 2 receptor (MC2R) at the adrenal gland (see Chapter 2).

The neonatal overfeeding effect on the brainstem catecholamine cells is clearly influenced by adult diet. Under chow-fed conditions the VLM response to LPS was exacerbated in the neonatally overfed, but responses on a background of HFD (3 days or 3 weeks) were similar. From our Chapter 4 results, we found neonatal overfeeding might actually be protective against the later effects of HFD. Although, the neonatal overfeeding causes body weight to be increased into adulthood, there was only a slight effect on the metabolic health of the animal and metabolic responses to an obesogenic diet. Furthermore, neonatal overfeeding, while increasing the PVN response to LPS in those rats fed chow as adults, significantly suppressed it in those fed 3 days HFD. Here we see a similar effect on the VLM. Neonatal overfeeding increased the VLM response to LPS in those rats fed chow as adults, but after 3 days and 3 weeks HFD there was no further increase. In Chapter 4, we investigated several forebrain regions that might have been involved in modulating the effects of stress on PVN in the neonatally overfed, including ventral bed nucleus of the stria terminalis (vBNST), ventromedial preoptic area (VMPOA), and vascular organ of the lamina terminalis (OVLT). In these regions we found no differences between any of the groups in their responses to LPS either with or without the 3 days and 3 weeks HFD. These findings suggest the possibility that neonatal overfeeding and HFD are acting directly at the VLM and NTS to disrupt (exacerbate) its response to LPS or that the PVN's feedback to the brainstem is disrupted, while the forebrain regions assessed remain unchanged.

Based on the results from above, we conclude the neonatal overfeeding does not change the response of total TH positive cells in NTS to LPS, but will increase TH cell activation in VLM, under chow-fed conditions; while on the background of a HFD the responses to LPS are similar. This means neonatal overfeeding can modify the NTS and VLM responses to LPS under certain dietary conditions. NTS and VLM send and receive projections to and from the PVN and contribute to the HPA axis response to stressors.

Neonatal overfeeding may thus change HPA axis responses to stress via a mechanism involving these brainstem regions. Future studies will need to consider the sex differences between the animals and if non-catecholamine cells in the brainstem are also affected by neonatal overfeeding.

Chapter 6

General Discussion

6.1 Major outcomes

The main hypothesis of this investigation was that neonatal nutrition programs the development of the stress response in rats. We aimed to test this by 1) Analysing if the responses of the stress axis are altered by neonatal overfeeding, 2) Analysing the effects of high fat diet on hypothalamic-pituitary-adrenal (HPA) axis responses to lipopolysaccharide (LPS) in neonatally overfed rats.

With the work presented in this thesis we found the central HPA axis response to LPS in neonatally overfed male rats tends to be normal. The sensitivity of the adrenal glands to downstream activation by LPS was reduced, however. Thus, neonatally overfed males had a suppressed LPS-induced and melanocortin 2 receptor (MC2R)-mediated release of glucocorticoids (GCs) from the adrenal glands. This slower GC release led to less efficient GC negative feedback on the HPA axis (Figure 6.1) and is likely linked to less efficient suppression of the nuclear factor κB (NFκB)-mediated transcription of cytokines (Stefanidis and Spencer 2012). For the high fat diet (HFD) study, the neonatally overfed males had different ventrolateral medulla (VLM) responses to LPS under HFD conditions, as well as elevated paraventricular nucleus of the hypothalamus (PVN) Fos responses to LPS and these effects were suppressed by 3 days HFD. LPS did not change the response of total tyrosine hydroxylase (TH) positive cells in NTS of males, but increased TH cell activation in VLM under chow-fed conditions, while on the background of a HFD the responses to LPS were similar. In females, the change in the mineralocorticoid receptor (MR) / glucocorticoid receptor (GR) ratio at the hippocampus is likely to have contributed to changes in HPA axis function (Figure 6.1).

Figure 6.1 HPA axis functions in neonatally overfed rat

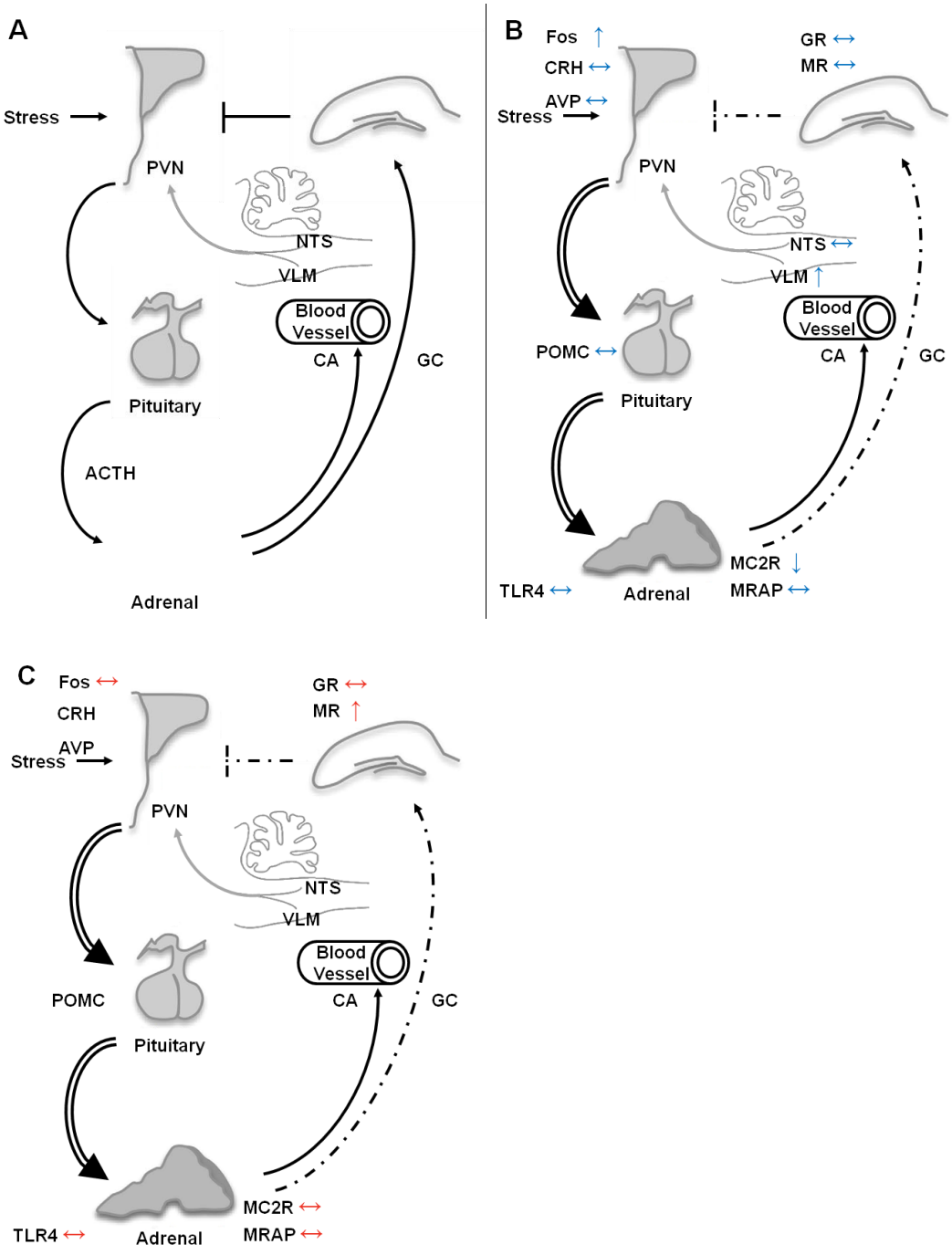


Figure 6.1 A) Under normal conditions, lipopolysaccharide (LPS) acts at the level of the brain to stimulate hypothalamic-pituitary-adrenal (HPA) axis activation, stimulating pituitary activation, releasing adrenocorticotropic hormone (ACTH), and activating adrenal glands to release glucocorticoid (GC). GC feeds back centrally to suppress further HPA axis activation. Catecholamine neurons (CA) activated in the nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM) project to the paraventricular nucleus of the hypothalamus (PVN), modulating this response. B) In neonatally overfed males central HPA axis responses to LPS are likely to be normal, stimulating PVN activation and ACTH release from the pituitary. The ability of

GCs to suppress further HPA axis activity at the level of the brain is also normal. However, the effect of ACTH on the adrenal is impaired, leading to slower LPS-induced activation of MC2R-mediated GC release, slower GC negative feedback and exaggerated PVN neuronal activation. Blue arrows indicate direction of gene differences between control (CL) and small litter (SL) groups after LPS treatment. The neonatally overfed males' NTS TH cell responses to LPS are similar to controls, but VLM TH cells are hyper-active in response to LPS. C) In neonatally overfed females, central HPA axis responses to LPS are likely to be normal, stimulating PVN activation and ACTH release from the pituitary. The MC2R is not changed in female adrenals. GC negative feedback and PVN neuronal activation are also tending to normal. However, the effect of ACTH on the adrenal is impaired leading to slower LPS-induced activation of MC2R-mediated GC release, slower GC negative feedback and exaggerated PVN neuronal activation. Red arrows indicate the direction of gene differences between CL and SL groups after LPS treatment. CRH: corticotropin releasing hormone; AVP: arginine vasopressin; POMC: pro-opiomelanocortin; TLR4: toll-like receptor 4; MC2R: melanocortin 2 receptor; MRAP: melanocortin receptor accessory protein; GR: glucocorticoid receptor; MR: mineralocorticoid receptor.

Overall these data indicate that neonatal overfeeding leads to long-term changes in the way male and female rats respond to bacterial endotoxin. These data also suggest the effects of neonatal overfeeding on male and female rat responses to an immune challenge may be through different pathways. The neonatal overfeeding attenuates acute central pro-inflammatory effects of short-term HFD in males and changes brainstem responses to LPS, but there are no similar effects on females. This may be related to the oestrous cycle stage effects on female responses that lead to variability in the female data. As above, the neonatal overfeeding influences on female HPA axis are likely to occur by mechanisms that are different from those in males.

6.2 Sex differences in HPA axis function

The HPA axis responses to stress are different between males and females, as has been shown by differences in the responses of stress-related chemicals such as ACTH and cortisol / corticosterone in human and rodent studies (Verma, Balhara et al. 2011). In some preclinical studies, for instance, the response to stress in female rodents is higher than in

males (Kitay 1963, Verma, Balhara et al. 2011). Men have higher ACTH levels with similar total cortisol concentrations under basal conditions compared with women, but women have a more sensitive adrenal cortex response to stress (Roelfsema, van den Berg et al. 1993). No differences were seen between males and females at the level of the pituitary when stimulated with synthetic human CRH, with or without dexamethasone pre-treatment (Siler-Khodr, Kang et al. 1997). However, the plasma ACTH response to ovine CRH was higher in women than in men (Born, Ditschuneit et al. 1995).

In our study, we see that the single dose of LPS significantly activated the PVN region in male rats, but not females in control animals. This result is in accordance with previous studies that have shown LPS activates the PVN (Conde, Renshaw et al. 1999) and that there are sex differences in this response (Seale, Wood et al. 2004). We also see the LPS injection led to a dramatic plasma corticosterone increase in control male rats at 60 and 90 minutes, but control female rats had elevated corticosterone at 90 and 120 minutes. These results show the males and females response to LPS stress may have temporal differences. We also found the control male rats' MC2R gene expression is increased by LPS application, but this does not occur in females. By contrast, the potential for GC negative feedback in the hippocampus is increased after LPS injection in females (in that the MR / GR ratio was lower in females after LPS), but we do not see similar results in males. Thus, these data indicate the mechanism of HPA axis responses to stress and GC negative feedback between males and females are different. These differences may be due, in part, to female's sex hormones and menstrual / oestrus cycle.

From previous studies, we know female sex hormones and menstrual / oestrus cycle can affect the HPA and sympatho-adrenal-medullary (SAM) axis responsiveness to stress. A functional magnetic resonance imaging study showed significant differences in activation of

HPA axis in adult women with activation attenuated during ovulation and increased during the early follicular phase, suggesting oestrogen can slow cortisol feedback on the brain and lead to a reduced or delayed stress response (Goldstein, Jerram et al. 2005, Verma, Balhara et al. 2011). Removal of the ovaries leads to a weakened HPA response and the oestrogens can increase the activity of HPA axis in animal studies (Stroud, Salovey et al. 2002). With the ovaries removed or in neonatally oestrogenised female rats there is an increased HPA axis response to stress and this is related to circulating gonadal steroid levels (Patchev and Almeida 1998). Rat ACTH and corticosterone levels are the highest around the time of ovulation, but in humans the changes of the HPA axis during the menstrual cycle are complex and there is still need for further research (Abplanalp, Livingston et al. 1977, Carey, Deterd et al. 1995). In the present investigation, we did not control for oestrous cycle stage because the necessary daily testing leads to significant stress in the rats (Liu, Diorio et al. 1997). We believe the impact on our results was minimal because similar variability was seen in females and in males in response to stress formost of our outputs. However, we also see the standard error of the mean for control male PVN neuronal activation in response to LPS was smaller than in females. Thus, this indicates the effects of the female menstrual / oestrous cycle on HPA axis function need to be considered in studies of stress.

6.3 Sex differences in early life programming

It is clear from the literature that stress can affect adult males and females differently (Verma, Balhara et al. 2011, Nelson and Lenz 2017, Perry, Goldstein-Piekarski et al. 2017). The sex differences have also been observed in response to environmental risk factors, such as early-life stress (Bale and Epperson 2015). From molecular studies, we see that the sex chromosome expressed can lead to differences in brain development between male and female responses to stress (Bale 2015, Nugent, Wright et al. 2015). Additionally, in

pregnancy the female rat exposes the male foetal brain to female hormones, such as oestrogen, via central aromatization of testosterone, and can set the steps of sex differences throughout life, however maternal stress can affect this procedure to interfere with full establishment of the male brain (Nugent, Wright et al. 2015). In male mice that were exposed to stress, their sperm microRNA content was different from males that were not stress-exposed, and their offspring behaviours and corticosterone responses to stress were also affected (Dietz and Nestler 2012, Rodgers, Morgan et al. 2013). Rodgers et al's study showed paternal stress can influence their offspring with some sex differences. For instance, corticosterone was lower in the male offspring of stressed sires compared with female offspring; male offspring had higher gene expression of CRH receptor 1 in pituitary and 11β-hydroxysteroid dehydrogenase (11β-HSD) in adrenals, but lower gene expression of pro-opiomelanocortin (POMC) in pituitary and MC2R in adrenals compared with females (Rodgers, Morgan et al. 2013). In mice studies, animals exposed to stress in pregnancy on embryonic days 1 to 7, the adult male, but not female, offspring had decreased activity in tail suspension and forced swim tests, and had lower scores in spatial learning and memory tasks (Mueller and Bale 2007, Mueller and Bale 2008). The male offspring also had increased plasma corticosterone levels in response to the acute restraint stress, as well as increased CRH in amygdala and reduced hippocampus GR gene expression (Mueller and Bale 2008).

Human males and females also have different HPA axis changes stimulated by early life stress (Maniam, Antoniadis et al. 2014). Male and female infants' salivary cortisol were similar in their median levels, but girls had a higher maximal level than boys (Tornhage 2002). Jones's study showed in 7 – 9 year old boys and girls, their salivary cortisol responses to stress were different. Girls' peak cortisol levels in the morning were inversely correlated with birth body weight, whereas, in boys, cortisol responses to stress but no morning cortisol levels were inversely correlated with birth body weight (Jones, Godfrey et al. 2006).

Early life factors additional to stress can also impact differently on the life of males and females. The studies from Roseboom's laboratory on victims of the Dutch famine showed that different nutritional environments *in utero* can influence the adults' responses to stress and as well as increase risk of several diseases. For instance, hypertension in men exposed to famine *in utero* in associated with large placental size whereas placental size tends to be small in men not exposed to *in utero* famine. In women, hypertension is independent of placental size (van Abeelen, de Rooij et al. 2011). Men *in utero* exposed during famine also had a significantly lower grip strength and a lower physical performance score than unexposed men, but again, there was no relationship in women (Bleker, de Rooij et al. 2016). Furthermore, men and women exposed *in utero* to famine, pass traits differently to their offspring: i.e. children from prenatally under-nourished fathers, are heavier and more likely to be obese than children from prenatally under-nourished mothers (Veenendaal, Painter et al. 2013).

Studies from other groups investigating neonatal overfeeding in rodents have also shown sex differences. After postnatal day 10, neonatally overfed females had a higher increase of inguinal fat than neonatally overfed males, and males had more expression of leptin gene and serum insulin than females (Argente-Arizon, Ros et al. 2016). These data also align with our group's previous work that neonatally underfed male (but not female) rats have reduced anxiety-related behaviour compared with their control siblings when tested in the elevated plus maze; they also have reduced activation of the PVN in response to the psychological stress, restraint, and corticosterone responses to restraint that recover more quickly than controls to baseline (Bulfin, Clarke et al. 2011). In female rats, neonatal overfeeding in early life can exacerbate HPA axis responses to psychological stress long-term, including PVN neuronal activation, but responses to psychological stress are not affected in males (Clarke, Stefanidis et al. 2012). As we have shown in this thesis, a likely

mechanism by which males are hyperactive to stress is through suppressed MC2R expression, whereas in females, the MC2R is normal and HPA axis responses are possibly different due to MR over-expression. Considering sex differences in HPA axis responses the stress is therefore important in future studies of early life programing.

6.4 The effects of 3 days and 3 weeks HFD

One of the more surprising outcomes from this investigation was that HFD did not exacerbate the phenotype caused by neonatal overfeeding. The results showed the plasma and liver triglyceride levels are increased overall with no effect of neonatal overfeeding. The inflammatory cytokines in liver and fat were also not affected by neonatal overfeeding in either males or females. 3 days HFD led to CL male rats having a six-fold increase in neuronal activation in the PVN after LPS-stress compared with chow-fed CL males. CL females had a similar increased response to LPS in numbers of PVN activated cells, but the response to LPS in PVN in females was not as robust. Although the pattern was not as clear as for the PVN, LPS did not change numbers of TH activated cells in NTS, however, LPS significantly increased the activation of TH cells in VLM of the neonatally overfed male rats after chow but not 3 days and 3 weeks HFD.

Short term HFD (1 week) can lead to vascular inflammation, and long term (8+ weeks) HFD can cause an inflammatory response in the liver, muscle and adipose (Kim, Pham et al. 2008). 3 days HFD also can cause hypothalamic microgliosis in rats and an increase pro-inflammatory gene expression, but the changes disappear after 7 days and reappear by 21 days (Thaler, Yi et al. 2012). However, we see the HFD did not affect inflammatory cytokines in adipose or liver in any of our results. These data indicate the HFD we chose for our study did not cause significant inflammation. The 3 days and 3 weeks HFD did increase PVN microglial activation in control males compared with chow-fed males (Cai,

Dinan et al. 2014), indicating the short-term and long-term HFD can directly affect the central immune system. Moreover, our results showed the neonatally overfed male rats have further increased PVN microglial activation after 3 weeks HFD (Cai, Dinan et al. 2014), suggesting the neonatal overfeeding can also influence central inflammation. It is noteworthy that different formulas of HFD and different periods of high fat feeding can affect the stress responses of animals. Maric et al.'s reported no differences between chow and HFD (the 32% kilocalories as fat) for 8 weeks, and only butter based HFD increased the fat pad and the total body weights in Wistar rats, with no effect of the coconut oil-based diet (Maric, Woodside et al. 2014). We also found in our model that responses to HFD are sex-dependent. The male rat seems more easily to be affected by 3 days HFD with the increase neuronal activation in PVN, but there was no specific effect of the diet on females. Female rats may thus be less sensitive than males to the negative effects of short-term HFD.

In this study, we have shown neonatal overfeeding led to a response to LPS that was six-fold lower than the male controls in their neuronal activation in PVN after a short-term HFD. Moreover, this response is somewhat different between females and males. The ability of neonatally overfed males to adjust to HFD appears to be stronger than that of control males. In addition, elevated hypothalamic inflammatory markers were found in rodents within 1 to 3 days of HFD, before the animals had weight gain. This is unlike the peripheral inflammation that develops as a consequence of obesity (Thaler, Yi et al. 2012). After only 1 week of HFD, the rodent hypothalamic arcuate nucleus had already sustained injury. However, with continued HFD, these responses temporarily subside. Neuroprotective mechanisms in the brain may thus initially limit the damage, with inflammation and gliosis returning to the mediobasal hypothalamus by 3 weeks (Thaler, Yi et al. 2012). Moreover, our group has seen that the GC release was unusually slowed in SL males (Clarke, Stefanidis et al. 2012), potentially indicating the SL are protected from central inflammation. The

mechanisms for this are complex and still need to be studied.

6.5 LPS as a stressor

The large part of this thesis has been investigating responses of the neonatally overfed to LPS. LPS certainly activates the HPA axis, but it also activates the global immune system. We found the liver Toll-like receptor (TLR)4 was increased in both groups by 3 days HFD. Translocated LPS stimulates TLR4 on Kupffer cells to release interleukin (IL) 1β, which induces hepatocyte steatosis and apoptosis, and ultimately activates hepatic stellate cells, and results liver fibrosis (Yang and Seki 2012). There are other types of stressor that activate the HPA axis without affecting the immune profile, such as physical stressors: i.v. isotonic blood volume expansion, alcohol, and haemorrhage and psychological stressors restraint, noise and forced swim (Dayas, Buller et al. 2001, Dayas, Buller et al. 2001, Dayas, Buller et al. 2004, Godino, Giusti-Paiva et al. 2005). The transition to alcohol dependence can cause injury of HPA axis functions and increase CRH levels outside the hypothalamus (Stephens and Wand 2012).

As part of the HPA axis response to psychosocial and physiological stressors, the GC released can induce several specific immune responses. For example, GC can inhibit the major type 1 cytokines, interferon-α, and IL-2 produced by T helper cell 1. Type 2 cytokines, IL-4, and IL-10 are not typically changed with increased GC. Thus, cell-mediated immunity is inhibited by GC and tends to shift to a type 2 cytokine pattern (Agarwal and Marshall 1998). However, psychosocial stress can decrease cellular immune function, and these differences were consistent in both human males and females but did not correlate with morning cortisol levels (Marshall, Agarwal et al. 1998). Thus, the specific role of GC in neonatal overfeeding succeptibility to inflammation remains to be determined.

6.6 Future directions

From the results from Chapter 2, we see the central HPA axis response to LPS in the neonatally overfed rats seems normal, but the sensitivity of the response to the ACTH in the adrenal gland is changed. Thus, neonatal overfeeding leads to a suppression of the MC2R-mediated response to ACTH and a suppressed increase in expression of MC2R. LPS results in slower GC release in the neonatally overfed compared with controls and then slower GC negative feedback to suppress the response. ACTH leads to GC responses that are nearly restored by 15 minutes in the CL (Redei, Li et al. 1994), but the SL response to ACTH is impaired and less efficient. Recent studies report that non-genomic intra-adrenal neegatire feedback and suppress ACTH-mediated GC release acts quickly within just a few minutes (Walker, Spiga et al. 2015) and might provide a mechanism to explain the differences we see in the neonatally overfed. However, the exact changes behind these molecular mechanisms remain to be discovered. In future studies it will be useful to examine kinetics and binding efficiency of ACTH on adrenal MC2R in response to stress.

The TLR4-induced myeloid differentiation primary response gene 88 (MyD88) pathway is an important inflammatory response in an animal's defence against bacterial infection (Lu, Yeh et al. 2008). On activation of the MyD88-dependent pathway, phosphorylation of IκB (inhibitor of κ light chain gene enhancer in B cells) proteins induces IκB kinase α (IKKα), IKKβ, and IKKγ phosphorylation leading to translocation of NFκB to the cell's nucleus and transcription of proinflammatory cytokines (Chang and Karin 2001). From our study, we tested the effect of TLR4 gene expression after LPS stress in our model and found no differences, but to determine the different target points of MyD88 pathway to responses to stress in neonatally overfed animals would be interesting to further clarify this aspect. We hypothesise the MyD88 pathway may be affected by neonatal overfeeding.

The results from Chapter 3 showed neonatal overfeeding is likely to change negative feedback of GC in the hippocampus in female rats, but the pituitary responses to LPS are not different. The mechanisms behind the differences in female HPA axis function after neonatal overfeeding are slightly different from those in males, but the ultimate differences are similar to males in that the GC response was slowed and suppressed. Thus, neonatal overfeeding modifies female HPA axis long-term and the mechanism by which this occurs is likely different from males. In males, an insensitivity of the MC2R to LPS may account for slower GC release, but in females, the different MR / GR ratio at the hippocampus may be the main change by which neonatal overfeeding has these effects. The female sex hormones and menstrual / oestrus cycle may lead to the differences between males and females, and be linked to these differences in HPA axis responses to stress (Stroud, Salovey et al. 2002). During the early follicular phase activation of HPA axis is likely to be increased, with a sharp peak before or during ovulation, then HPA axis activation will slowly decrease throughout the luteal phase, the basal GC level are the highest around this time (Carey, Deterd et al. 1995). Moreover, oestrogen can slow human cortisol feedback on the female brain and lead to a reduced or delayed stress response (Goldstein, Jerram et al. 2005, Verma, Balhara et al. 2011). However, our findings showed minimal differences in within-group variability between males and females to suggest that oestrous cycle differences may contribute to these effects. Thus, the question remains, how are female sex hormones and menstrual / oestrus cycle affecting the SAM and HPA axis responsiveness to stress after neonatal overfeeding? To test this, in future studies we will track the female cycle and analyse the effects of cycle stage on SAM and HPA axis to the stress responses. Moreover, if cycle stage and female hormones are playing a role in the effects of neonatal overfeeding on the HPA axis we should be able to restore normal responses with overiectomy and oestrogen replacement therapy.

From Chapter 4 we see the responses of the neonatally overfed rats to HFD are different between males and females. Although, HFD led to males and females neuronal activation in the PVN being increased after LPS injection, the responses in PVN between males and females are slightly different. Male rats (but not females) are more likely to be affected by 3 days HFD with increase PVN neuronal activation if they have been overfed as neonates (Cai, Dinan et al. 2014). Short-term HFD either of saturated or unsaturated fat leads to male mice (but not females) developing insulin resistance (Senthil Kumar, Shen et al. 2014). Similarly, male rats given high fructose or sucrose diet, had insulin resistance and hypertension, but females did not (Galipeau, Verma et al. 2002). Thus, female rats may be less sensitive to the negative effects of short-term HFD than males. Thus, female rats made overweight by neonatal overfeeding are unlikely to be substantially more vulnerable to a short-term adult-onset HFD than normals in terms of developing further obesity or a diabetogenic profile. For future studies it will be interesting to repeat our present experiments but give the controls and neonatally overfed rats different types of diet, such as high fructose, sucrose, or saturated fats. It will also be interesting to assess the results of a longer duration of HFD. We hypothesize this will have a more detrimental effect on the neonatally overfed than on the controls.

As we have described previously, our response to HFD in the neonatally overfed are sex-dependent. The male rat seems more easily to be affected by LPS with the increase neuronal activation in PVN, but there was no specific effect on the females. Female rats may thus be less sensitive than males to the negative effects of stressors. Monitoring female cycle stage would give females an additional stress (Lovick 2012), so we did not do this. However, we believe the cycle stage may have affected our experiments. We can see the female results are more variable than males. These also showed the comparisons only have sex difference but no statistic post hoc difference between groups. There are also very few studies in the

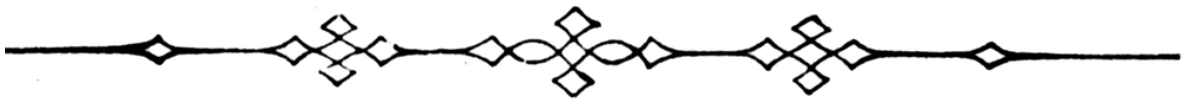
literature reporting data from both males and females in this area of research. When provided a short-term HFD either of saturated or unsaturated fat, male mice had insulin resistance, but females did not (Senthil Kumar, Shen et al. 2014). High fructose or sucrose feeding can make the male rats insulin resistant, as well as hypertensive, but this effect is not seen in females (Galipeau, Verma et al. 2002). Thus, female rats may be less immune sensitive than males to the effects of LPS due to complex physiological mechanisms. Further work to extend these studies will give more ideas to help to understand the differences between sexes.

The neonatal overfeeding effect on the brainstem catecholamine cells is clearly influenced by adult diet. In Chapter 5, under chow-fed conditions the VLM response to LPS was exacerbated by the neonatally overfed, but responses on a background of HFD (3 days or 3 weeks) were similar. We see neonatal overfeeding leads to male rats increasing the VLM response to LPS if they were fed chow as adults, and significantly suppressing it in those fed 3 days HFD. However, the NTS response to LPS did not change. NTS and VLM send and receive projections to the PVN and contribute to the HPA axis response to stressors (Smith and Vale 2006). Thus, these data suggest that neonatal overfeeding and HFD are possibly directly acting at the VLM and NTS to exacerbate the response to LPS, or the feedback from PVN to brainstem is disrupted in male rats. It would be interesting to examine if the female neonatally overfed rats have similar brainstem responses to bacterial endotoxin. In order to analyse if the projections between PVN and brainstem are directly affected, it would be interesting to employ neuronal tract tracing techniques. Some basic methods are include anterograde and retrograde labelling, for instance with autoradiography with radiolabelled amino acid and retrograde fluorescence tracer (Oztas 2003). We can also use a fluorescence-based double retrograde tracer to compare the projection patterns to different brain areas (Apps and Ruigrok 2007). We hypothesise the neonatal overfeeding can weaken the immune responses to stress and permanently change projection between PVN of the central HPA and

NTS and VLM of the SAM axis, and the HFD can increase this effect.

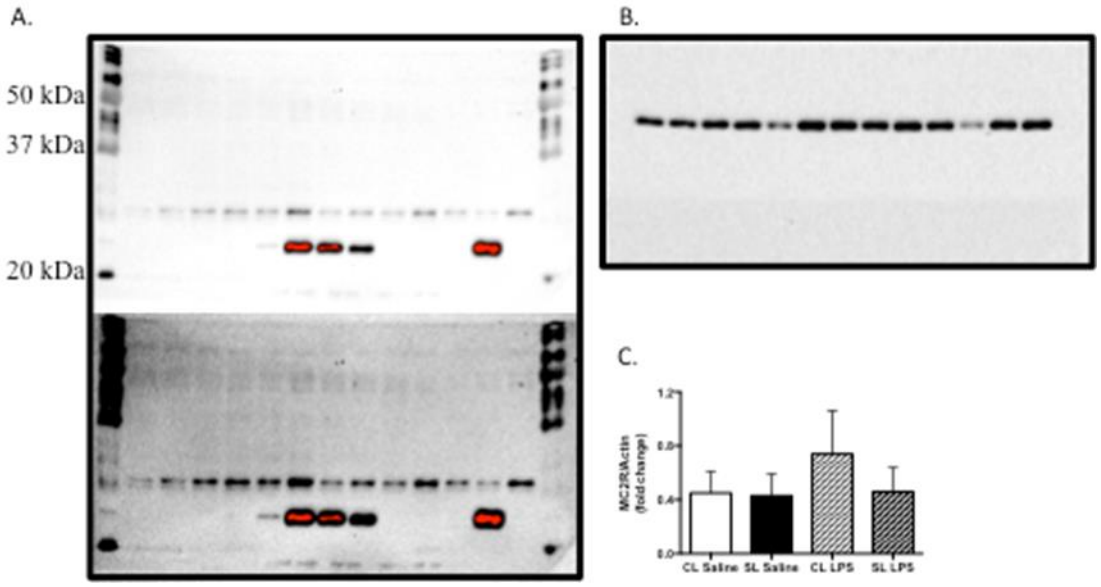
6.7 Summary

Based on our results, we conclude neonatal overfeeding leads to rat HPA axis responses to stress in males and females that are exacerbated and prolonged. In males, we see the neonatal overfeeding leads to reduced efficiency of the adrenal response to endotoxin, and the capability of the rat to respond to the infections is inhibited, probably due to changes in the efficiency of the MC2R response to LPS. In females a similar end-profile is seen, but this is likely due to changes in sensitivity of GC negative feedback at the hippocampus. Neonatally overfed are not substantially more vulnerable to 3 days or 3 weeks HFD but the neonatal overfeeding can change the responses to LPS in NTS and VLM in SAM axis under certain dietary situations. These data suggest neonatal overfeeding leads to reduced efficiency in an animal's ability to combat an immune challenge but does not substantially impair their response to a short-term HFD. Here we show important mechanistic effects for these changes and highlight some novel sex differences in the long-term effects of neonatal overfeeding. These research findings are potentially useful for the education and information for the public on appropriate nutrition. Balanced nutritional intake should be considered peri-pregnancy and post-partum to avoid overweight infants. Also, these data may shift the research focus from the adult overweight and obese to further investigation into childhood obesity, with the goal to prevent obesity throughout life. The hope is that intervening in childhood obesity would reduce the resources spent on overweight and obese and their related disease costs from the public health service.



Supplementary Data

Supplementary 1 Adrenal western blot results



Supplementary 1 A – C) MC2R Protein level in adrenal glands. A) Depicts an 8 second exposure during which very strong bands became visible at 25 kDa and weaker bands visible at approximately 30 kDa (i.e. non-target). Even when severely overexposed (lower image), there are still no visible bands at the expected 42 kDa. B) Depicts actin on the stripped membrane at the expected 44 kDa. C) Depicts densitometry on the ~30 kDa bands on this and two other blots containing the remaining samples. MC2R Protein level in adrenal glands did not show any difference (p = 0.812).

Appendices

Appendix 1 Overfeeding during a critical postnatal period exacerbates hypothalamic-pituitary-adrenal axis responses to immune challenge: a role for adrenal melanocortin 2 receptors

SCIENTIFIC REPORTS

OPEN

Overfeeding during a critical postnatal period exacerbates hypothalamic-pituitary-adrenal axis responses to immune challenge: a role for adrenal melanocortin 2 receptors

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Early life diet can critically program hypothalamic-pituitary-adrenal (HPA) axis function. We have previously shown rats that are overfed as neonates have exacerbated pro-inflammatory responses to immune challenge with lipopolysaccharide (LPS), in part by altering HPA axis responses, but how this occurs is unknown. Here we examined neonatal overfeeding-induced changes in gene expression in each step of the HPA axis. We saw no differences in glucocorticoid or mineralocorticoid receptor expression in key regions responsible for glucocorticoid negative feedback to the brain and no differences in expression of key HPA axis regulatory genes in the paraventricular nucleus of the hypothalamus or pituitary. On the other hand, expression of the adrenal melanocortin 2 receptor (MC2R) is elevated after LPS in control rats, but significantly less so in the neonatally overfed. The *in vitro* adrenal response to ACTH is also dampened in these rats, while the *in vivo* response to ACTH does not resolve as efficiently as it does in controls. These data suggest neonatal diet affects the efficiency of the adrenally-mediated response to LPS, potentially influencing how neonatally overfed rats combat bacterial infection.

Obesity is characterised by a chronic low-grade inflammatory profile that contributes to leptin resistance and the onset of diabetic symptoms[1]. This altered inflammatory profile can also lead to an increased susceptibility to and morbidity and mortality from infections[2]. For instance, obese patients are twice as likely to develop pneumonia, up to six times as likely to contract a post-surgical infection, and 2.1 times as likely to die in intensive care due to infection-related complications than normal-weight patients are[2,3]. Our recent findings suggest the hypothalamic-pituitary-adrenal (HPA) axis may play an important role in regulating this susceptibility[4].

We have recently demonstrated, in rodents, an overweight/obese phenotype that occurs as a result of overfeeding in early life leads to a markedly exacerbated neuroimmune response to the bacterial mimetic, lipopolysaccharide (LPS)[4]. Neonatally overfed rats have increased white adipose tissue expression of pathogen-associated molecular pattern recognition receptor toll like receptors (TLR)4 and 2. They have increased phosphorylation of inhibitory factor κB, elevated circulating pro-inflammatory cytokine levels, and higher fevers after LPS compared with rats from a control background. This response is likely to be at least partly due to macrophage proliferation within adipocytes and a subsequent elevated expression of TLR4 leading to enhanced downstream effects of the LPS in these rats. However, findings from this study also suggest these neonatally overfed rats have important changes in their HPA axis responses to LPS[4,5].

The HPA axis is the body's principal endocrine response to stress, including to immune challenge[6,7]. HPA axis activation culminates in glucocorticoid (GC) release into circulation, which acts at immune cells to interfere

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with nuclear factor κB-mediated transcription of pro-inflammatory cytokines, thus attenuating fever[8]. We have seen neuronal activation in the paraventricular nucleus of the hypothalamus (PVN; as measured by immunohistochemistry for Fos) is markedly increased after LPS in neonatally overfed rats compared with controls, as was anticipated based on the pro-inflammatory cytokine and febrile responses[4,5]. Interestingly, however, circulating corticosterone concentrations are not simply higher after LPS in the obese. Neonatal overfeeding is associated with a slower corticosterone response so that the peak is reached significantly later after exposure to LPS than in controls[4]. These findings imply an inefficiency in HPA axis negative feedback may contribute to an exacerbated response to an immune challenge.

The HPA axis is remarkably sensitive to early life programming events. For instance, high intensity parenting (high levels of licking and grooming accompanied by significant arched-back nursing) in the rat leads to long-term increases in glucocorticoid receptor (GR) gene expression in the hippocampus resulting in attenuated HPA axis responses to stress[9–12]. In this line, humans with a history of abuse during childhood have reduced hippocampal GR gene expression and exacerbated responses to stress throughout life, as well as increased susceptibility to illness; effects that are mediated through epigenetic mechanisms[12–15]. The HPA axis is also influenced by early life immune experience with neonatal immune challenge leading to an exacerbated HPA axis responses to an immune challenge of the same type despite a suppression of the febrile and sickness behaviour components of the response[16,17]. In this case, the mechanism likely involves hypersensitivity / hyper-expression of liver and spleen TLRs leading to early activation of the HPA axis through an early liver-derived increase in prostaglandins[16].

In the present investigation, we aimed to examine changes in HPA axis function in male rats made obese due to neonatal overfeeding to determine if alterations in GC sensitivity or negative feedback are involved in the exacerbated response to LPS seen in these animals. Here we induced neonatal overfeeding by manipulating the litter sizes in which the rat pups are suckled, generating litters of 4 (small litter; SL; neonatal overfeeding) and 12 (control litter; CL). When the rats reached adulthood, we examined changes in neuronal activation and gene expression in several steps of the HPA axis that were likely to be influenced by neonatal overfeeding.

Results

Neonatal overfeeding enhances short- and long-term weight gain. As we have previously seen[18–20], neonatal overfeeding (SL) is associated with accelerated growth during the suckling period ($F_{(3,66)} = 30.46$, $p < 0.001$) such that SL were significantly heavier than CL by postnatal day (P)7 and this was maintained through to P14 and P21 (n = 8 litters each; Fig. 1A). This weight phenotype was maintained into adulthood ($F_{(1,90)} = 23.30$, $p < 0.001$) and the groups allocated to saline or LPS treatments were not different from one another (Fig. 1B).

Neonatal overfeeding exacerbates HPA axis responses to LPS. We have previously reported that male rats raised in SL have more neurons activated in the PVN in response to a single injection of LPS in adulthood than those raised in CL, as assessed by numbers of Fos-immunoreactive cells in the region[4,5]. They also have differences in their GC response to the LPS[4]. We replicate those findings here in that LPS led to significant activation of the PVN in SL but not CL rats and there were significantly more Fos-positive cells in the PVN in SL than CL after LPS (significant litter size x LPS interaction; $F_{(1,19)} = 9.46$, $p = 0.006$; n = 5–7; Fig. 1C,E). Here LPS also led to a significant increase in CL plasma corticosterone that peaked at 60 min and had plateaued or was returning towards baseline by 90 and 120 min. In SL rats, the corticosterone response to LPS was slower and remained elevated at 120 min (litter size × LPS × time interaction; $F_{(4,128)} = 7.46$, $p < 0.001$; n = 6–13; Fig. 1D).

LPS effects on hippocampal GR expression in neonatally overfed rats. These exacerbated responses to LPS in neonatally overfed rats led us to examine what changes in the HPA axis may be responsible for these differences. When the HPA axis is activated, GC are released and these feed back onto GR in the hypothalamus and hippocampus to suppress further activity. We thus examined hypothalamic and hippocampal GR and mineralocorticoid (MR) expression under basal conditions and after an LPS challenge to assess the capacity for GC negative feedback in SL and CL rats (n = 7–8). In the hypothalamus there was no effect of litter size or LPS on GR or MR expression and no effect when GR mRNA was expressed as a ratio to MR (Fig. 2A–C). In the hippocampus, there was a significant effect of LPS ($F_{(1,26)} = 10.11$, $p = 0.004$), with LPS increasing GR expression in SL but not CL (Fig. 2D). LPS and neonatal overfeeding did not affect hippocampal MR or MR/GR ratios (Fig. 2E,F).

LPS effects on hypothalamic corticotropin-releasing hormone in neonatally overfed rats. We next examined potential changes in the expression of various genes known to regulate HPA axis function at the level of the hypothalamus and pituitary (n = 7–8 as above). In these experiments SL rats had reduced corticotropin-releasing hormone (CRH) mRNA after LPS compared with saline (significant effect of LPS ($F_{(1,29)} = 7.34$, $p = 0.012$; Fig. 2G). Arginine vasopressin (AVP) mRNA levels were similar between all the groups (Fig. 2H). There was a significant effect of litter size on pituitary pro-opiomelanocortin (POMC) expression ($F_{(1,28)} = 5.35$, $p = 0.028$), with SL having reduced POMC expression compared with CL, but there were no differences with *post hoc* tests (Fig. 2I).

The LPS-induced increase in melanocortin 2 receptor adrenal gene expression is suppressed in neonatally overfed rats. When the HPA axis is stimulated, adrenocorticotropic hormone (ACTH) released from the anterior pituitary acts at the melanocortin 2 receptor (MC2R) on the adrenal cortex to stimulate GC production and release. We therefore next examined adrenal expression of MC2R and its accessory protein, melanocortin receptor accessory protein (MRAP; n = 7–8 as above). There were no significant effects on absolute or percentage adrenal weights (Fig. 3A, B). LPS significantly elevated adrenal MC2R mRNA by 2 hr after injection in CL and SL rats (litter size x LPS interaction; $F_{(1,26)} = 8.94$, $p = 0.006$), but this effect was significantly less robust in

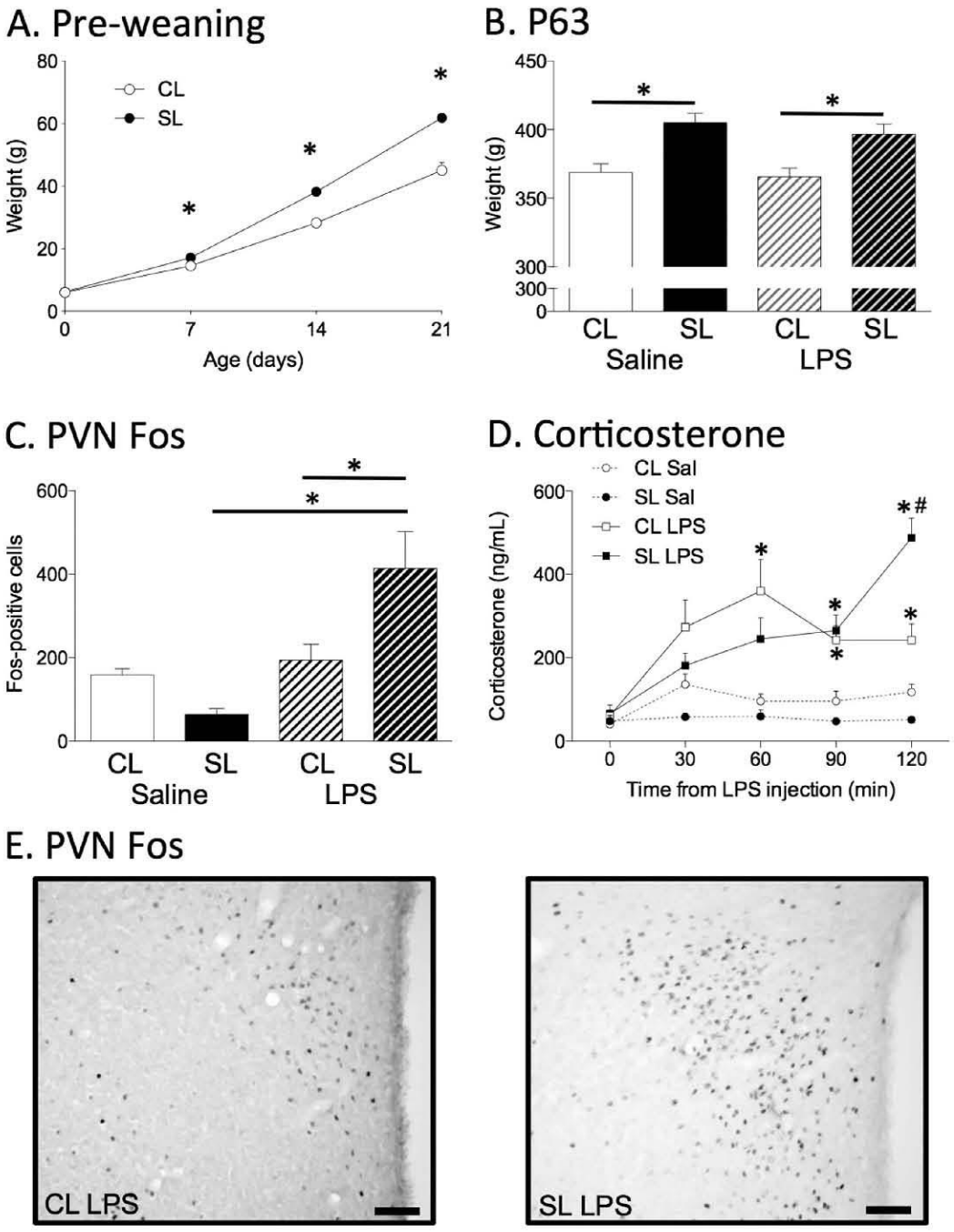

Figure 1. (**A**) Pre-weaning and (**B**) Adult (postnatal day (P)63) body weights of rats raised in control (CL) and small (SL) litters. (**C**) Paraventricular nucleus of the hypothalamus (PVN) neuronal activation (Fos) in response to i.p. LPS. (**D**) Plasma corticosterone in response to i.p. LPS. (**E**) Photomicrographs of the PVN from representative CL and SL LPS-treated rats. Scale = 100 μm. Data are mean + SEM. *compared with saline-treated group for the same litter size, #compared with CL-LPS; $p < 0.05$.

the SL group compared with CL (Fig. 3C). We saw no significant differences in MRAP gene expression between the groups but the main effect of litter size on gene expression was $p = 0.072$ (Fig. 3D).

Neonatally overfed rats have impaired *in vitro* release and inefficient *in vivo* suppression of ACTH-stimulated corticosterone. Our findings outlined above were indicating neonatally overfed rats had differences in the ability of the adrenal cortex to respond to the ACTH generated as part of the HPA axis response to LPS. To investigate if this was the case, we measured the *in vitro* effect of ACTH on the adrenal release of corticosterone. *In vitro,* ACTH stimulated significant adrenal corticosterone release in CL but not SL rats (significant effect of ACTH; $F_{(3,24)} = 9.70$, $p < 0.001$; Fig. 4A; n = 6 per group), with a significant difference seen

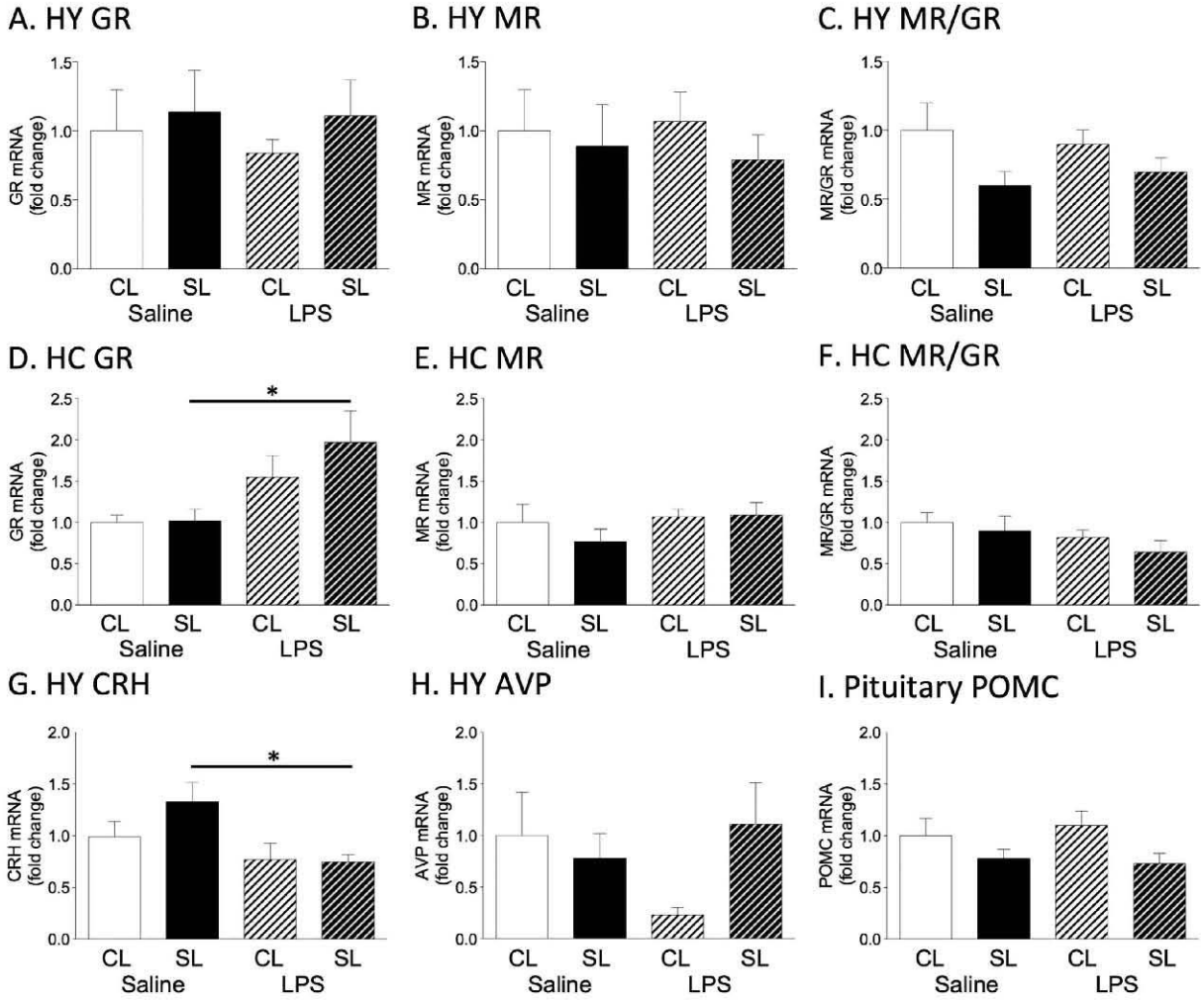

Figure 2. Hypothalamic (**A**–**C**) and hippocampal (**D**–**F**) expression of (**A**,**D**) glucocorticoid receptor (GR), (**B**,**E**) mineralocorticoid receptor (MR), (**C**,**F**) MR/GR ratio (**G**) hypothalamic expression of corticotropin-releasing hormone (CRH), (**H**) hypothalamic expression of arginine vasopressin (AVP), (**I**) pituitary expression of pro-opiomelanocortin (POMC) of adult rats raised in control (CL) and small (SL) litters 2 hr after i.p. LPS. Data are mean + SEM. $^*p < 0.05$.

30 min after exposure to ACTH. These findings indicated neonatal overfeeding leaves the rats less able to respond to stimulation of the HPA axis, likely due to their reduced ability to increase expression of the MC2R.

To test if this was the case *in vivo*, we also measured plasma corticosterone responses to ACTH injection in CL and SL rats. In this experiment we found the corticosterone response to ACTH was small and not significantly different from the response to saline in CL rats. In SL, corticosterone was significantly increased relative to saline at 30 min after ACTH injection, indicative of a robust adrenal response in these rats (significant time × ACTH interaction; $F_{(4,128)} = 7.93, p < 0.001$; time × ACTH interaction; $F_{(4,128)} = 2.31, p < 0.061$; n = 9 per group; Fig. 4B).

To test if the differences between the *in vivo* responses to LPS and to ACTH were due to a direct effect of LPS at the adrenal gland we also stimulated adrenals *in vitro* with LPS and examined corticosterone responses. *In vitro* LPS mildly suppressed adrenal corticosterone release in CL (significant effect of ACTH; $F_{(3,24)} = 5.61, p = 0.005$; Fig. 4C; n = 6 per group), with significant differences from baseline seen at 30 and 45 min after LPS exposure, but there were no effects in SL adrenals. Neonatal overfeeding also did not alter adrenal expression of TLR4 (Fig. 4D).

Discussion

Early life events have established and pronounced effects on HPA axis function. Here we present the first evidence that the adrenal MC2R may be involved in the response, with early life overfeeding altering the LPS-induced expression of this receptor. Thus, as we have previously reported, male rats made overweight long-term by early life overfeeding have exacerbated central (hypothalamic) neuronal activation in response to LPS[4,5]. In a similar profile to our previous findings, the GC response in the neonatally overfed is slower, with corticosterone taking longer to reach a peak and return to baseline[4]. In accordance with this finding, expression of the MC2R gene is significantly and substantially elevated after LPS in rats fed a control diet as neonates, but in the neonatally overfed this response is comparatively suppressed.

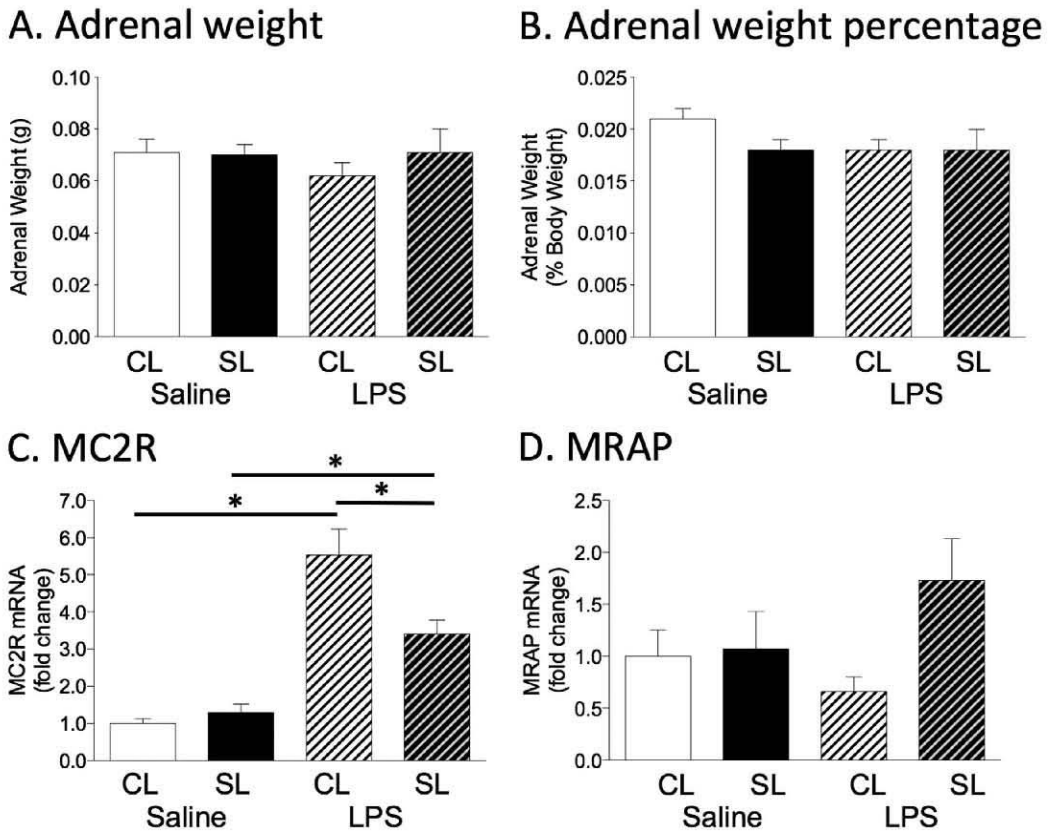

Figure 3. (**A**) Absolute adrenal weight, (**B**) adrenal weight expressed as a percentage of total body weight, (**C**) adrenal gland expression of melanocortin 2 receptor (MC2R) mRNA, (**D**) adrenal expression of melanocortin receptor accessory protein (MRAP) mRNA of adult rats raised in control (CL) and small (SL) litters 2 hr after i.p. LPS. Data are mean + SEM. $^*p < 0.05$.

Upon exposure to LPS, cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)α and IL-6, stimulate activation of the PVN leading to downstream ACTH release from the pituitary, which interacts with MC2R and leads to GC release from the adrenal. Indeed, in neonatally overfed animals, the cytokine response to LPS is exacerbated, likely leading to enhanced stimulation of the HPA axis at its apex[4]. LPS-stimulated GC normally inhibit further pro-inflammatory cytokine transcription and feed back directly and indirectly onto the PVN to inhibit further HPA axis activation and GC release[21,22]. Our observation that the GC response to LPS is slower and the PVN response exacerbated in SL rats led us to hypothesise GC negative feedback would be less efficient at the level of the hypothalamus or hippocampus as has been previously seen with models of maternal care[9–12]. Unlike in rats given low levels of maternal care as neonates, whose hippocampal GR expression is suppressed leading to exacerbated HPA axis responses to stress, neonatal overfeeding induced no such changes in GR expression. Although hippocampal GR expression was significantly increased in SL rats and not CL after LPS, there was no significant difference between the CL and SL groups in this response. There was also no difference between the groups in MR expression or the MR:GR ratio, important for assessment of MR:GR balance as an indicator of HPA axis function[23,24]. It is therefore unlikely neonatal overfeeding is influencing the HPA axis response to LPS by altering negative feedback at the hypothalamus and hippocampus. It is noteworthy that these early studies by Meaney and colleagues describe an excellent relationship between GR gene and protein expression[25], as does our previous study with early life immune challenge[16]. As we saw no differences between the CL and SL groups in gene expression, we did not follow up with assessment of the protein. As with GC negative feedback, the ability of the PVN CRH cells to respond to LPS is probably also intact in that neonatal overfeeding had no effect on CRH or AVP expression either under basal conditions or after LPS, except that LPS suppressed CRH expression in SL but not significantly in CL. Pituitary POMC expression was likewise not different between the groups, indicating the ability to produce ACTH in response to a stimulus is probably not affected by neonatal diet.

Neonatal diet did, however, significantly affect adrenal MC2R expression in response to LPS. It would be very interesting to examine if MC2R protein levels reflect the changes in gene expression we see. However, we could not eliminate non-specific bands with the commercially available antibodies (data not shown). Others have also reported that there is currently no suitable antibody for this protein[26]. This change in MC2R gene expression at least implies the SL adrenal may be less able to efficiently respond to LPS-induced ACTH to stimulate GC release. From this finding, we hypothesized that the GC response to ACTH alone would also be attenuated in SL rats and this was indeed the case in our *in vitro* preparation. ACTH administrated *in vivo* to neonatally overfed rats actually led to more GC release at 30 min after injection in these than in controls, potentially reflecting differences in

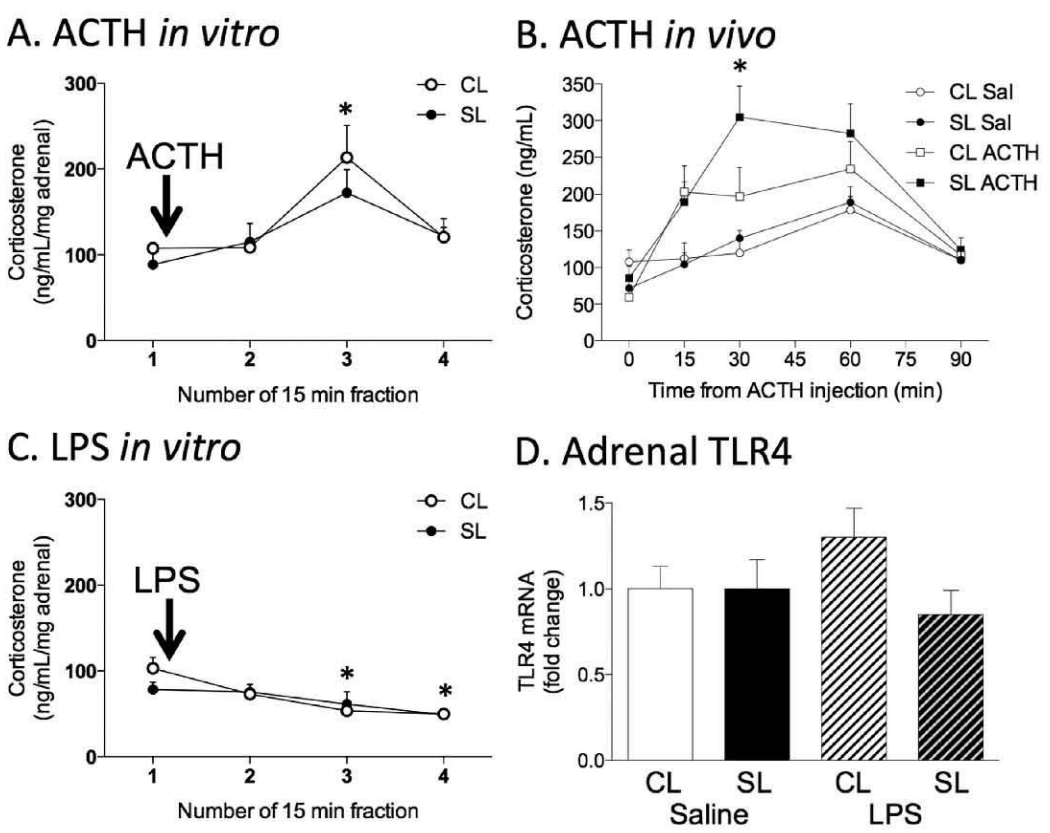

Figure 4. (**A**) Adrenocorticotropic hormone (ACTH)-induced corticosterone in adrenals from adult rats raised in control (CL) and small (SL) litters. ACTH (10^{-7} M added after collection of fraction 1). (**B**) ACTH-induced plasma corticosterone in adult CL and SL rats. (**C**) Lipopolysaccharide (LPS)-induced adrenal corticosterone. LPS (1 μg/mL added after collection of fraction 1). (**D**) Adrenal gland toll-like receptor (TLR)4 mRNA. Data are mean + SEM. (**A**) *compared with fraction 1, 2, and 4 for CL; $p < 0.05$, corrected for adrenal weight. (**B**) *compared with saline-treated group for the same litter size; $p < 0.05$. (**C**) *compared with fraction 1 for CL; $p < 0.05$, corrected for adrenal weight.

the time course of the response to LPS versus ACTH alone or implying LPS might act directly at the adrenal to dampen GC release.

Although there is little published data on the adrenal MC2R and how LPS may affect it, there is some suggestion that LPS may directly influence the MC2R-mediated GC response. LPS can suppress the ACTH-induced GC response in fasciculata reticularis and glomerulosa cells[27,28]. Prior exposure to LPS can induce endotoxin tolerance in the adrenal, with the corticosterone response to LPS or ACTH being suppressed in primary fasciculata reticularis cells from rats that had previously been given LPS[29]. Importantly, MC2R−/− mice do not mount a GC response to LPS (or to restraint stress), indicating that MC2R is necessary for both the ACTH-mediated and direct LPS-mediated GC response[30]. The MC2R is a G protein-coupled receptor whose activation is dependent upon the presence of the accessory protein MRAP. The activation of MC2R leads to conversion of ATP into cAMP via stimulation of adenylyl cyclase. Activation of protein kinase A ensues and leads to phosphorylation of cAMP response element protein, activating the transcription of steroidogenic acute regulatory protein (StAR) and other genes involved in steroidogenesis[31,32]. There is some evidence LPS can interact with this mechanism, modifying the binding of ACTH to the cell membrane and modifying the ACTH signal transduction pathway, suppressing ACTH-induced cAMP production in primary culture[27]. However, it is unlikely the differences we see between controls and neonatally overfed are due to a direct action of LPS at the level of the adrenal. Expression of adrenal TLR4 was not affected by litter size. Additionally, while LPS led to a suppression of corticosterone after 30 and 45 min in CL in our *in vitro* preparation, no such effects were seen in SL.

Collectively, our data strongly suggest the central HPA axis response to LPS in neonatally overfed rats is probably normal, but that there is a change in the sensitivity of the adrenal gland to the ACTH released after HPA axis activation such that its direct effects are modified (Fig. 5). Thus, neonatal overfeeding leads to a suppression of the MC2R-mediated response to ACTH and a suppressed increase in expression of MC2R. In the *in vivo* presence of LPS this is seen as slower GC release and thus slower GC negative feedback to suppress the response. In the case of a single stimulus with ACTH, it is likely the GC response is already nearly resolved by 15 min in the CL[33], but the SL response is again impaired or less efficient. The differences in the time courses of these stimuli (LPS and ACTH alone) may explain the differences in the profiles of the corticosterone release. It is noteworthy in this regard that non-genomic intra-adrenal negative feedback to suppress ACTH-mediated GC release has recently been identified and this acts very rapidly, i.e. within minutes[34]. The exact molecular mechanisms behind the changes we see

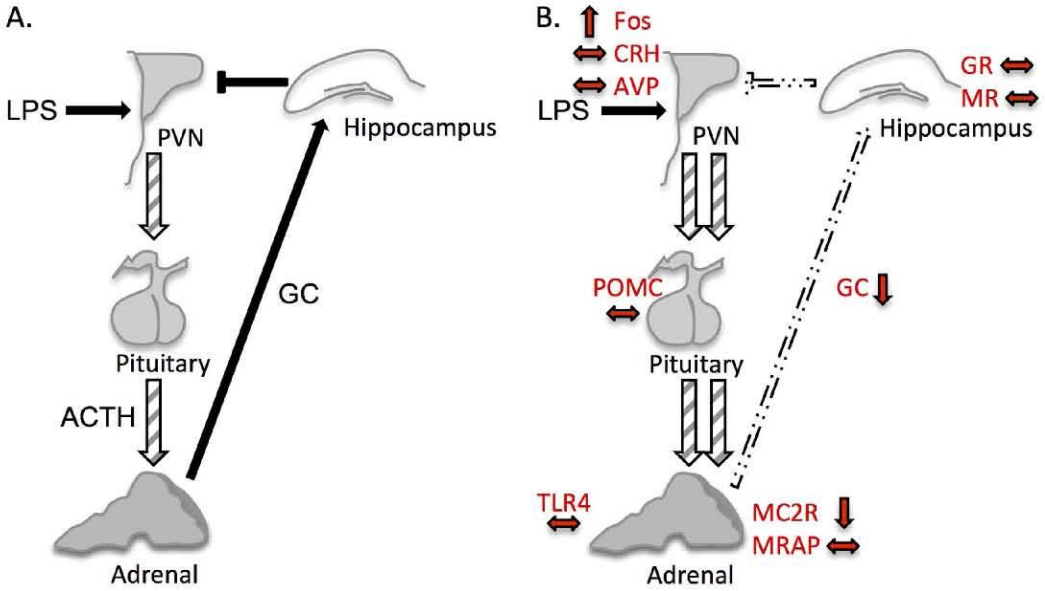

Figure 5. Proposed circuitry by which neonatal overfeeding leads to exacerbated hypothalamic-pituitary-adrenal (HPA) axis responses to lipopolysaccharide (LPS). (**A**) Under normal conditions LPS acts at the level of the brain to stimulate HPA axis activation and glucocorticoid (GC) production. GC feed back centrally to dampen further HPA axis activation. (**B**) In neonatally overfed rats, central HPA axis responses to LPS are likely to be normal, stimulating paraventricular nucleus of the hypothalamus (PVN) activation and adrenocorticotropic hormone (ACTH) release from the pituitary. The ability of GC to suppress further HPA axis activity at the level of the brain is also normal. However, the effect of ACTH on the adrenal is impaired leading to slower LPS-induced activation of MC2R-mediated GC release, slower GC negative feedback and exaggerated PVN neuronal activation. Red arrows indicate direction of gene differences between CL and SL groups after LPS treatment. AVP, arginine vasopressin; CRH, corticotropin releasing hormone; GC, glucocorticoids; GR, glucocorticoid receptor; MC2R, melanocortin 2 receptor; MR, mineralocorticoid receptor; MRAP, melanocortin receptor accessory protein; POMC, pro-opiomelanocortin.

remain to be determined, but the implication of these data and that they have exacerbated febrile and cytokine profiles after LPS[4], is that neonatally overfed animals have a less efficient adrenally-mediated response to bacterial endotoxin. In this regard, neonatally overfed rats may therefore be less able to combat bacterial infection. There is also the possibility that this inefficiency could be compensated for with supplementary GC, an idea that also requires further study.

Methods

Animals. We obtained timed pregnant Wistar rats from the Animal Resources Centre, WA, Australia. They were maintained at 22 °C on a 12 hr light / dark cycle (0700–1900 hr) with pelleted rat chow and water available *ad libitum*. All procedures were conducted in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals and were approved by the RMIT Animal Ethics Committee.

Litter manipulation. On the day of birth (P0) we removed all the pups from their dams and randomly reallocated them to new dams in litters of 4 or 12 as we have described previously[18–20]. Care was taken that no dam received any of her own pups. Each new litter was made up of 50% males and 50% females. Excess pups were culled. Females were used in other experiments.

Following pup reallocation, the litters were weighed weekly as whole litter units; we have previously shown that males and females show similar growth rates until after weaning[35]. At weaning the pups were separated into same-sex littermate pairs where they were left undisturbed, except for the usual animal husbandry, until experimentation. All experimental groups were derived from 3 or more litters, using a maximum of 2 pups from the same litter for an experimental treatment. N are indicated in the results.

HPA axis responses to immune challenge with i.p. LPS. At approximately P70, we took each rat from its cage and nicked the end off the tail with a sharp razor blade to extract a ~20 μL baseline blood sample into a heparinized capillary tube. We collected each sample within 3 min of nicking the tail to minimize any handling effects on the corticosterone levels detected in the sample[36]. We then gave each rat an i.p. injection of LPS (*Escherichia coli*, serotype 026:B6; L-3755; Sigma, St Louis, MO, USA; 100 μg/kg in 1 mg/kg pyrogen-free saline), or pyrogen-free saline and took blood samples at 30, 60, 90, and 120 min after injection. Blood samples were kept on ice until the end of the experiment, when they were centrifuged and the plasma aliquots stored at −20 °C until assayed.

Target Gene	NCBI Reference Sequence	TaqMan Assay ID	Product Size
Nr3c1	NM_012576.2	Rn00561369_m1	73
Nr3c2	NM_013131.1	Rn00565562_m1	79
Crh	NM_031019.1	Rn01462137_m1	112
Avp	NM_016992.2	Rn00690189_g1	78
Pomc	NM_139326.2	Rn00595020_m1	92
Mc2r	NM_001100491.1	Rn02082290_s1	126
Mrap	NM_001135834.1	Rn01477212_m1	62
Tlr4	NM_019178.1	Rn00569848_m1	127
18s	X03205.1	4319413E	187

Table 1. TaqMan probe details (Life Technologies) used for qRT-PCR.

At 120 min after injection, and immediately after the final blood sample, we deeply anaesthetized the rats with Lethabarb (~150 mg/kg pentobarbitone sodium, i.p.) and perfused them transcardially with phosphate buffered saline (PBS; 4 °C, pH 7.4) followed by 4% paraformaldehyde in PBS (4 °C, pH 7.4) for collection of fixed brains. We then removed the brains and post-fixed them for 4 hr in the same fixative before placing them in cryoprotectant with 20% sucrose in PBS (4 °C). We subsequently cut forebrains into 40 μm coronal sections using a cryostat. All experiments were initiated between 0900 and 1200 hr to limit potential effects of circadian rhythms on any parameters measured.

Fos immunohistochemistry. We assessed neuronal activation in the fixed brains on the basis of positive Fos-immunoreactivity, seen as a black deposit in the nucleus. Briefly, a one-in-four series of forebrain sections from each animal was incubated in primary Fos antibody (overnight; 4 °C; 1:10 000; rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), then in secondary antibody (90 min; room temperature; 1:200; biotinylated anti-rabbit; Vector Laboratories, Burlingame, CA, USA) and in an avidin-biotin horseradish peroxidase (HRP) complex (1 hr; Vector Elite kit, Vector). The sections were then incubated in nickel diaminobenzidine to visualize the HRP activity, seen as a black nuclear deposit. The reaction was terminated once an optimal contrast between specific cellular and non-specific background labelling was reached. Sections from each treatment group were processed simultaneously. Sections were mounted on chrome-alum-coated slides, dehydrated in a series of alcohols, cleared in Histoclear and coverslipped.

Cell counts. An experimenter, blinded to the group treatments, carried out counts of cells positive for Fos-immunoreactivity in the PVN. These were counted over two sections (~1.8 and 1.96 mm caudal to bregma).

Corticosterone assays. We measured plasma corticosterone concentrations using a standard rat corticosterone ELISA (Abnova Corp., Taipei, Taiwan). The inter-assay variability for this assay was 7.2% coefficient of variation (CV), intra-assay variability 4.8% CV, and lower limit of detection 40 pg/mL. We assayed samples from all treatment groups together in duplicate.

Real time reverse transcriptase polymerase chain reaction (rt-PCR) analysis. To determine candidate steps in the HPA axis that were altered by neonatal overfeeding, we also took a cohort of CL and SL male rats at P70 and collected fresh tissue for rt-PCR at 2 hr after LPS (or saline) injection. We deeply anaesthetised the rats with Lethabarb and quickly removed hypothalamus, hippocampus, pituitary, and adrenal glands over ice. We weighed adrenals, immediately snap-froze all tissues in liquid nitrogen, and stored them at −80 °C until use.

We isolated RNA from brain, pituitary, and adrenals using QIAzol and an RNeasy purification kit (QIAGEN, Valencia, CA, USA). RNA (1 μg) was transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturers' instructions. Real-time rt-PCR was performed using Taqman Gene Expression Assays (Table 1; Applied Biosystems, Mulgrave, Vic, Au). Fold differences in target mRNA expression were measured using the delta-cycle threshold method by comparison with the housekeeping gene, 18S[37,38] and expressed as mRNA relative fold change as described previously[16,39].

Corticosterone responses to adrenocorticotropic hormone. As our data suggested the effect of neonatal overfeeding on HPA axis function was principally due to changes in the adrenal gland, we examined corticosterone responses to stimulation of the adrenal *in vivo* with ACTH (1.5 μg/kg in 1 mL/kg pyrogen-free saline, s.c). We gave CL and SL adult rats ACTH and took blood samples immediately prior to injection and 15, 30, 60, and 90 min after injection. Samples were analysed as above.

Adrenal corticosterone responses *in vitro*. To assess the *in vitro* effect of ACTH and LPS on the adrenal release of corticosterone, we deeply anaesthetised CL and SL rats with Lethabarb, excised their adrenal glands and stored the adrenals in ice-cold Dulbecco's modified Eagle's medium/Nutrient mixture F-12 (DMEM/F-12; Thermo Fisher Scientific, Scoresby, Victoria, Australia) containing 0.1% BSA until all tissues were collected. We then bisected each adrenal gland, weighed and pre-incubated each half adrenal gland for two × 1 hr in 1 mL of DMEM/F-12 at 37 °C in a 95% O_2/5% CO_2 atmosphere. After the pre-incubation period, we refreshed the medium and collected samples every 15 min. ACTH (10^{-7} M)- and LPS (1 μg/mL)-containing medium was added in the second fraction. At the end of each 15 min period the medium was collected and stored in −20 °C until

assayed for corticosterone levels using a standard rat corticosterone ELISA, as described above. The protocol was adapted from[40–42] and we determined doses in pilot experiments.

Data analysis. We compared pre-weaning body weights between CL and SL rats using an analysis of variance (ANOVA) with repeated measures, with litter size as the between factor and age as the repeated measure. When a significant interaction was found between litter size and age, we performed Student's unpaired t-tests for each time point. We compared adult weights, Fos-positive cell counts and gene expression using two-way ANOVAs with litter size and LPS-treatment as between factors. Corticosterone concentrations were compared using repeated measures ANOVAs with litter size and LPS- or ACTH-treatment as the between factors and time as the repeated measure. We used Tukey *post hoc* comparisons where significant interactions were found. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was assumed when $P < 0.05$.

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Author Contributions

Authors G.C. and S.J.S. designed the study and conducted statistical analyses. G.C., I.Z., J.B., A.S., L.S., J.C.M. and S.J.S. conducted the experiments. S.J.S. analysed the data and wrote the manuscript. G.C., I.Z., J.B., A.S., L.S., J.C.M. and S.J.S. all contributed to and have approved the final manuscript.

Additional Information

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Appendix 2 Neonatal overfeeding attenuates acute central pro-inflammatory effects of short-term high fat diet

frontiers in NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
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Neonatal overfeeding attenuates acute central pro-inflammatory effects of short-term high fat diet

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Neonatal obesity predisposes individuals to obesity throughout life. In rats, neonatal overfeeding also leads to early accelerated weight gain that persists into adulthood. The phenotype is associated with dysfunction in a number of systems including paraventricular nucleus of the hypothalamus (PVN) responses to psychological and immune stressors. However, in many cases weight gain in neonatally overfed rats stabilizes in early adulthood so the animal does not become more obese as it ages. Here we examined if neonatal overfeeding by suckling rats in small litters predisposes them to exacerbated metabolic and central inflammatory disturbances if they are also given a high fat diet in later life. In adulthood we gave the rats normal chow, 3 days, or 3 weeks high fat diet (45% kcal from fat) and measured peripheral indices of metabolic disturbance. We also investigated hypothalamic microglial changes, as an index of central inflammation, as well as PVN responses to lipopolysaccharide (LPS). Surprisingly, neonatal overfeeding did not predispose rats to the metabolic effects of a high fat diet. Weight changes and glucose metabolism were unaffected by the early life experience. However, short term (3 day) high fat diet was associated with more microglia in the hypothalamus and a markedly exacerbated PVN response to LPS in control rats; effects not seen in the neonatally overfed. Our findings indicate neonatally overfed animals are not more susceptible to the adverse metabolic effects of a short-term high fat diet but may be less able to respond to the central effects.

Keywords: inflammation, microglia, neonatal, obesity, paraventricular nucleus of the hypothalamus (PVN)

INTRODUCTION

The developmental origins of health and disease hypothesis suggests the early life period is one of significant vulnerability to programming of physiology by environmental influences (Forsdahl, 1977; Barker and Osmond, 1986; Wadhwa et al., 2009; Spencer, 2012). In particular, early life nutrition is important in programming the development of central and peripheral mechanisms regulating feeding and metabolism, and subsequent susceptibility to overweight or obesity (Spencer, 2012, 2013a,b). As such, perinatal overfeeding has major short- and long-term physiological consequences [e.g., reviewed in (Spencer, 2012, 2013a; Habbout et al., 2013)].

We, and others, have reported neonatal overfeeding in a rodent model leads to accelerated weight gain in early life that persists long-term and is linked with immune and hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Plagemann et al., 1992; Boullu-Ciocca et al., 2005; Spencer and Tilbrook, 2009; Clarke et al., 2012; Smith and Spencer, 2012; Stefanidis and Spencer, 2012). These findings parallel those of human studies where childhood obesity significantly increases the risk an individual will become an obese adult (Whitaker et al., 1997; Stettler et al., 2005; Biro and Wien, 2010). Obese children are also more likely to suffer from immune and HPA axis disturbances as they grow up (Reeves et al., 2008; Lee, 2009; Brune and Hochberg, 2013).

Although there are clear effects of early life nutrition on later susceptibility to overweight/obesity and its pathophysiological sequelae, it is also clear not all overweight children become obese adults (Potter and Ulijaszek, 2013). Similarly, several studies of neonatal overfeeding in rodents have shown that long-term exacerbated weight gain is mild and the animals do not always exhibit hyperphagia or indices of diabetes. For instance, while some studies have demonstrated being suckled in small litters leads to increased food intake in adulthood (Oscai and McGarr, 1978; Rodrigues et al., 2007, 2009), this tends to normalize when corrected for overall body weight (Mozes et al., 2004; Xiao et al., 2007; Stefanidis and Spencer, 2012). Studies also differ in their reporting of whether neonatal overfeeding influences glucose utilization (Plagemann et al., 1999; Xiao et al., 2007). Although some neonatally overfed cohorts show insensitivity to a glucose load in a glucose tolerance test (GTT), differences in glucose uptake into adipocytes, and differences in insulin signaling (Plagemann et al., 1999; Boullu-Ciocca et al., 2005; Rodrigues et al., 2007), indicating a pre-diabetic phenotype, we have seen only mild changes in metabolic parameters (Stefanidis and Spencer, 2012). In this regard neonatal overfeeding appears to result in a moderate predisposition to excessive weight gain, with some indications of diabetic symptoms and significant, but mild, metabolic impairment.

From a pathophysiological perspective, a single adverse event or period is unlikely to be the only factor influencing long-term physiology, however. A sustained high fat diet consumed in adult rodents and humans can lead to excessive weight gain, adiposity, and indices of diabetes such as glucose intolerance and insulin resistance (Rosini et al., 2012). In this study we therefore hypothesized that the mild metabolic phenotype induced by neonatal overfeeding would predispose an animal to more substantial metabolic disturbances later in life if it is also exposed to the "second hit" challenge of a short or medium term high fat diet.

Neonatal overfeeding by suckling rat pups in small litters induces notable but moderate changes in weight gain, feeding, and metabolism throughout life that may be exacerbated by later exposure to high fat diet. However, neonatal overfeeding also causes significant and substantial peripheral and central inflammation, including a pro-inflammatory profile in systemic tissue and the hypothalamus, as well as exacerbated pro-inflammatory response to a neuroimmune challenge with bacterial mimetic lipopolysaccharide (LPS) (Tapia-Gonzalez et al., 2011; Clarke et al., 2012; Ye et al., 2012; Ziko et al., 2014). For this reason we also hypothesized the systemic and central inflammatory profile would be further exacerbated by high fat diet in adulthood in the neonatally overfed rats.

In this study we manipulated litter sizes so that Wistar rats were suckled in litters of four (small litter; SL) or 12 (control litter; CL). The former have greater access to their dam's milk, consume milk that is higher in fat, and show accelerated growth and weight gain that is maintained into adulthood (Fiorotto et al., 1991; Mozes et al., 2004). The pups were weaned onto *ad libitum* normal rat chow, but in adulthood were given either 3 days (3D) or 3 weeks (3W) high fat diet (45% kcal as fat). At the end of this period we assessed changes in weight and indices of diabetes, as well as central and peripheral markers of inflammation. We also examined liver cytokine expression and central neuronal activation in response to i.p. LPS.

MATERIALS AND METHODS

ANIMALS

We obtained timed-pregnant Wistar rats from the Animal Resources Centre, WA, Australia. After arrival at the RMIT University Animal Facility, we housed the dams at 22°C on a 12 h light/dark cycle (7 a.m. to 7 p.m.) with free access to pelleted rat chow and water. We conducted all experiments in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care of Experimental Animals. All procedures were approved by the RMIT University Animal Ethics Committee.

LITTER SIZE MANIPULATION

On postnatal day (P) 0, the day of birth, we removed all pups from their dams and randomly fostered them to new dams in litters of 12 (CL; controls) or 4 (SL; neonatally overfed) as we have previously described (Spencer and Tilbrook, 2009; Clarke et al., 2012; Smith and Spencer, 2012; Stefanidis and Spencer, 2012; Ziko et al., 2014). Birth litters included in this study had a range of 8–17 pups, a mean of 13.9 ± 0.36, and mode of 14. No dam received any of her own pups and each new litter was made up of 50% males and 50% females. Excess pups were culled. We have previously shown this litter size manipulation results in SL pups having accelerated growth and weight gain so that they are significantly heavier by around P7 and maintain greater weights into adulthood (Spencer and Tilbrook, 2009; Clarke et al., 2012; Smith and Spencer, 2012; Stefanidis and Spencer, 2012; Ziko et al., 2014).

EFFECTS OF NEONATAL OVERFEEDING ON SUSCEPTIBILITY TO HIGH FAT DIET

To test long-term susceptibility to the effects of high fat diet after neonatal overfeeding, we weaned the rats into same-sex littermate pairs on normal rat chow and kept them until P56. At this time they were allocated to the 3D or 3W high fat diet or chow groups. 3D high fat diet (23.5% fat; 45% kcal from fat; Specialty Feeds, WA, Au) was commenced at P74 and 3W high fat diet (as above) was commenced at P56. On P76, i.e., 2 days or 20 days after the onset of the high fat diet, or equivalent in chow fed (4.8% fat) controls, we gave the rats an i.p. glucose tolerance test (GTT). Rats were fasted for 3–4 h prior to testing to standardize basal glucose levels. We then quickly took each rat from its cage and nicked the end off the tail with a sharp razor blade to extract ~20 μL of baseline blood sample into a heparinized capillary tube for measurement of plasma triglycerides. These and liver triglycerides were later determined using calorimetric enzymatic GPO-PAP assays (Roche Diagnostics, IN, USA). Blood samples were kept on ice until the end of the experiment, when they were centrifuged and the plasma aliquots stored at −20°C until assayed. We also measured basal glucose levels at this time using an Accu-Chek Performa blood glucose meter (Roche Diagnostics; Castle Hill, NSW, Au). We then injected each rat with 1.5 g/kg glucose and measured glucose levels at 15, 30, 45, 60, and 90 min after injection.

Two days later, i.e., after 4 or 22 days high fat diet (or chow), the pairs of rats were then randomly allocated into the saline or LPS group. We gave each rat an i.p. injection of LPS (*E. coli*, serotype 026:B6; L-3755; Sigma, St Louis, MO, USA; 100 μg/kg), or pyrogen-free saline. At 120 min after injection, we deeply anesthetized the rats with Lethabarb (~150 mg/kg pentobarbitone sodium, i.p.). We hemisected each rat below the diaphragm and used it for fresh tissue collection and for cardiac perfusion to obtain fixed brains. Thus, we removed livers and male epididymal or female perirenal fat pads. Tissues were weighed and snap-frozen in liquid nitrogen. For the brains, we perfused the rats transcardially with phosphate buffered saline (PBS; 4°C, pH 7.4) followed by 4% paraformaldehyde in PBS (4°C, pH 7.4). We then removed the brains and post-fixed them for 4 h in the same fixative before placing them in cryoprotectant with 20% sucrose in PBS (4°C). We cut forebrains into 30 μm coronal sections using a cryostat. All experiments were initiated between 0900 and 1200 h to limit potential effects of circadian rhythms on any parameters measured.

INFLAMMATORY GENE EXPRESSION

To assess changes in peripheral markers of inflammation, we measured mRNA expression levels of the free fatty acid and

LPS receptor, toll-like receptor 4 (TLR4), downstream transcription factor, nuclear factor κB (NFκB), as well as representative pro- and anti-inflammatory cytokines, interleukin (IL)-10, tumor necrosis factor (TNF)α, IL-1β, and IL-6 in the liver and adipose tissues. We isolated RNA from our snap-frozen liver and fat samples using QIAzol and an RNeasy purification kit (QIAGEN, Valencia, CA, USA). The extracted RNA (1 μg) was transcribed to complementary DNA with an iScript cDNA synthesis kit; (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. We then performed rt-PCR with Taqman Gene Expression Assays (Applied Biosystems, Mulgrave, Vic, Au). We measured fold differences in target mRNA expression with the δ-cycle threshold method by comparison with the housekeeping gene, 18S (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Data are expressed as mRNA relative fold change as described previously (Mouihate et al., 2010; Clarke et al., 2012; Spencer et al., 2012).

LIVER CYTOKINE EXPRESSION

To further assess changes in peripheral markers of inflammation, we examined concentrations of a number of pro- and anti-inflammatory cytokines in the liver using a Bio-Plex assay allowing multiple analytes to be assessed in one sample. Liver samples were lysed using Bio-Plex cell lysis kit (Bio-Rad) according to the manufacturer's instructions. The total protein concentration of the lysates was determined using the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific). Samples were then diluted in Bio-Plex Sample Diluent (containing 0.5% BSA) and assayed in a final concentration of 500 ug/mL using a magnetic beads-based Bio-Plex Pro rat TH1/TH2 12-Plex (Bio-Rad) assay. The assays were performed using the Bio-Plex MAGPIX™ instrument and the data were analyzed using Bio-Plex Manager Software 6.1 (Bio-Rad). Female IL-13, granulocyte macrophage colony-simulating factor, and interferon gamma were not detectable and these were low and not significantly different between groups in the males, so are not reported here.

BRAIN MICROGLIA AND NEURONAL RESPONSES TO IMMUNE CHALLENGE

To assess the influence of early life overfeeding and adult high fat diet on central inflammation, we immunolabelled sections through the hypothalamus for ionized calcium-binding adapter molecule-1 (Iba-1; a marker for microglia/macrophages), seen as amber staining or Fos (a marker of neuronal activation), seen as a black nuclear deposit, as previously described (Spencer et al., 2004a,b; Mouihate et al., 2010; Ziko et al., 2014). Briefly, we incubated separate one-in-five series of forebrain sections from each animal in primary antibody overnight at 4°C (Iba-1, 1:1000; rabbit; Wako Chemicals USA Inc., Richmond, VA, USA or Fos, 1:10,000; rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by secondary antibody (1.5 h; 1:200, Iba-1, 1:500, Fos; biotinylated anti-rabbit; Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin horseradish peroxidase (HRP) complex (ABC; 45 min; Vector Elite kit; Vector). The sections were then incubated in diaminobenzidine (DAB) with (black; Fos) or without (amber; Iba1) nickel and colbalt, to visualize the HRP activity. The reactions were terminated once an optimal contrast between specific cellular and non-specific background labeling was reached. Randomly selected brains from each of the treatment groups were processed at the same time in batches. Sections were then air-dried, dehydrated in a series of alcohols, cleared in histolene, and coverslipped.

CELL COUNTS

An experimenter blinded to treatment condition assessed the sections for differences in numbers of cells with Iba-1 labeling and in density of Iba-1 labeling using photomicrograph images imported into image analysis software Image J (National Institutes of Health, Bethesda, MD, USA), as we have previously described (Beynon and Walker, 2012; Radler et al., 2014; Ziko et al., 2014). Briefly, we took all photomicrograph images from an Olympus upright microscope (Olympus BX41; Olympus, Melbourne, Vic, Au) with a 20 times objective lens using an Olympus DP72 digital camera (Olympus) and LabSens image capture software v1.6 (Olympus) software. Images were taken at 4140 × 3096 pixel density. They were then imported into and processed using Image J. We auto-subtracted background and converted each image to 16 bit for analysis, then cropped each image to take a representative 1602 × 1602 pixel sample from each region of interest within each section. We then assessed numbers of Iba-1-positive cells and density of staining using the thresholding method, as described (Beynon and Walker, 2012; Radler et al., 2014; Ziko et al., 2014), in the paraventricular nucleus of the hypothalamus (PVN; ∼1.80 and 1.95 mm caudal to bregma) and in the arcuate nucleus (ARC; ∼2.04 to −3.09 mm relative to bregma). Brain regions were identified according to the Paxinos and Watson Rat Brain Atlas (Paxinos and Watson, 2009). For each region, we sampled the left and right sides across two sections of the PVN and five sections of the ARC, 150 μm apart. We saw no differences between left and right hemispheres or rostrocaudal level for any of the regions, so we then took the sum of the images as our sampled result.

An experimenter, blinded to the group treatments, also carried out counts of cells positive for Fos-immunoreactivity in the PVN over two sections (∼1.80 and 1.95 mm caudal to bregma), in the dorsal (d) and ventral (v) bed nucleus of the stria terminalis (BNST) over four sections (∼0.24 to −0.36 mm relative to bregma), in the medial preoptic area (MPOA) and vascular organ of the laminar terminalis (OVLT) over two sections (∼0.36 and 0.51 mm rostral to bregma) and in the ventromedial (VM) POA over two sections (at and 0.15 mm caudal to bregma).

DATA ANALYSIS

We compared pre-weaning body weights between CL and SL rats using an analysis of variance (ANOVA) with repeated measures, with litter size as the between factor and age as the repeated measure. When a significant interaction was found between litter size and age we performed Student's unpaired *t*-tests for each time point. We compared adult parameters using multi-factorial ANOVAs with litter size, sex, adult diet, and LPS treatment as between factors where appropriate, with Tukey *post-hoc* comparisons where significant main effects or interactions were found. We also included time (min) as a repeated measure in analysis of

plasma glucose concentrations. Data are presented as the mean + standard error of the mean (SEM). Statistical significance was assumed when $P \leq 0.05$. Statistical details are reported in the figure legends.

RESULTS

WEIGHT GAIN WITH NEONATAL OVERFEEDING

As we, and others, have previously reported (Spencer and Tilbrook, 2009; Clarke et al., 2012; Ziko et al., 2014), being suckled in SL leads to accelerated weight gain and this is maintained into adulthood compared with rats from CL. Thus, being raised in SL led to pups being significantly heavier by as early as P7 and this was maintained throughout the suckling period (**Figure 1A**) and into adulthood (**Figure 1B**).

WEIGHT GAIN, FOOD INTAKE, AND CALORIC EFFICIENCY WITH HIGH FAT DIET IN ADULTHOOD

Neonatal overfeeding did not cause significant differences in the weight gained with the 3D high fat diet in males or females (**Figures 2A,E**). There were significant effects of sex and diet, with females gaining less weight over the period than males, and those on high fat diet gaining less weight than those on standard rat chow, but there were no differences between relevant groups with *post-hoc* comparisons. After 3W of high fat diet, all female groups had gained less weight than all male groups. There was also an effect of litter size, with SL gaining more weight than CL but no differences between relevant groups with *post-hoc* comparisons (**Figures 2I,M**).

Consistent with their size, females ate less than males in both the 3D and 3W analyses. There was also a significant effect of diet on food intake after 3W, with high fat diet-fed rats eating fewer grams of food than standard chow-fed rats, in total and for each of the 3 weeks (**Figures 2B,F,J,N**).

Calculations of total energy consumption revealed the high fat diet groups consumed more energy than the chow groups at 3D and 3W, and males ate more than females. However, there was no influence of neonatal overfeeding on total energy consumption (**Figures 2C,G,K,O**).

Caloric efficiency is a measure of the ability to convert calories into body weight. Thus, a reduced caloric efficiency reflects the need to consume more calories to maintain body weight. 3D high fat diet significantly reduced caloric efficiency in SL but not CL male and female rats (**Figures 2D,H**). The 3W high fat diet significantly reduced caloric efficiency in SL but not CL females (**Figures 2L,P**).

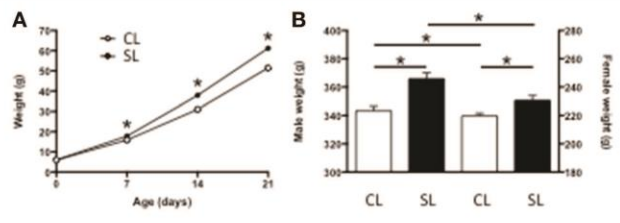

FIGURE 1 | Effects of neonatal overfeeding on body weight. (A) Pre-weaning (age, litter size interaction [$F_{(3,\ 78)} = 28.83$, $P < 0.001$] and **(B)** Adult (P56; significant effect of litter size [$F_{(1,\ 84)} = 25.96$, $P < 0.001$] and sex [$F_{(1,\ 84)} = 1559.46$, $P < 0.001$] body weights of rats raised in control (CL) and small (SL) litters. Data are mean + SEM. $^*P < 0.05$.

FAT MASS AND TRIGLYCERIDE CONTENT WITH HIGH FAT DIET IN ADULTHOOD

Surprisingly, there were also no differences in total or percentage fat between any of the CL and SL groups (**Figures 3A,B,E,F**). We did not make a sex comparison in this analysis since the fat pads were different. There was a significant effect of litter size on plasma triglyceride concentrations, with generally increased triglyceride levels in rats from SL. There was also an effect of sex, with females of each group having lower triglyceride levels than their male counterparts (**Figures 3C,G**). We also detected significant effects of litter size and diet on liver triglyceride concentrations, with SL and the high fat diets increasing these levels (**Figures 3D,H**).

GLUCOSE UTILIZATION WITH HIGH FAT DIET IN ADULTHOOD

In accordance with the minimal effects of the high fat diet seen on overt measures of weight gain and adiposity, we also saw no significant differences in fasting glucose levels, or tolerance to glucose among the groups in males or females (**Figure 4**).

PERIPHERAL INFLAMMATION WITH HIGH FAT DIET IN ADULTHOOD; GENE EXPRESSION

We have previously reported neonatal overfeeding influences peripheral and central immune profiles (Clarke et al., 2012; Ziko et al., 2014). We therefore tested if neonatal overfeeding exacerbates the peripheral and central response of inflammatory markers to high fat diet. In the liver there was an increase in TLR4 mRNA after 3D high fat diet in both CL and SL males compared with their chow-fed counterparts. Interestingly, this increase in TLR4 did not persist, but had returned toward baseline values after 3W (**Figure 5A**). There were no significant differences between the female groups with *post-hoc* tests and no sex differences, but CL females did show a tendency to have elevated TLR4 after 3D high fat diet compared with chow-fed females (**Figure 5B**).

In liver there was a significant effect of sex on NFκB, IL-10, and IL-1β mRNA, with females expressing more of these three genes than males, but there were no significant differences with *post-hoc* tests except in that there was more IL-1β in females after 3W high fat diet than in males. There were no differences between the groups in liver TNFα mRNA and IL-6 was undetectable in this tissue (**Figure 5**).

We analyzed male epididymal and female perirenal fat separately as the fat was taken from different regions. There was a significant effect of litter size on fat NFκB in the males, with SL having more NFκB than CL, but there were no significant differences between the individual groups with *post-hoc* tests (**Figure 5G**). There was also a significant effect of diet on male IL-10 and IL-1β with the high fat diets reducing expression of these cytokines, but again there were no *post-hoc* differences and no further significant differences in male or female fat TLR4, NFκB, TNFα, or IL-6 mRNA (**Figure 5**).

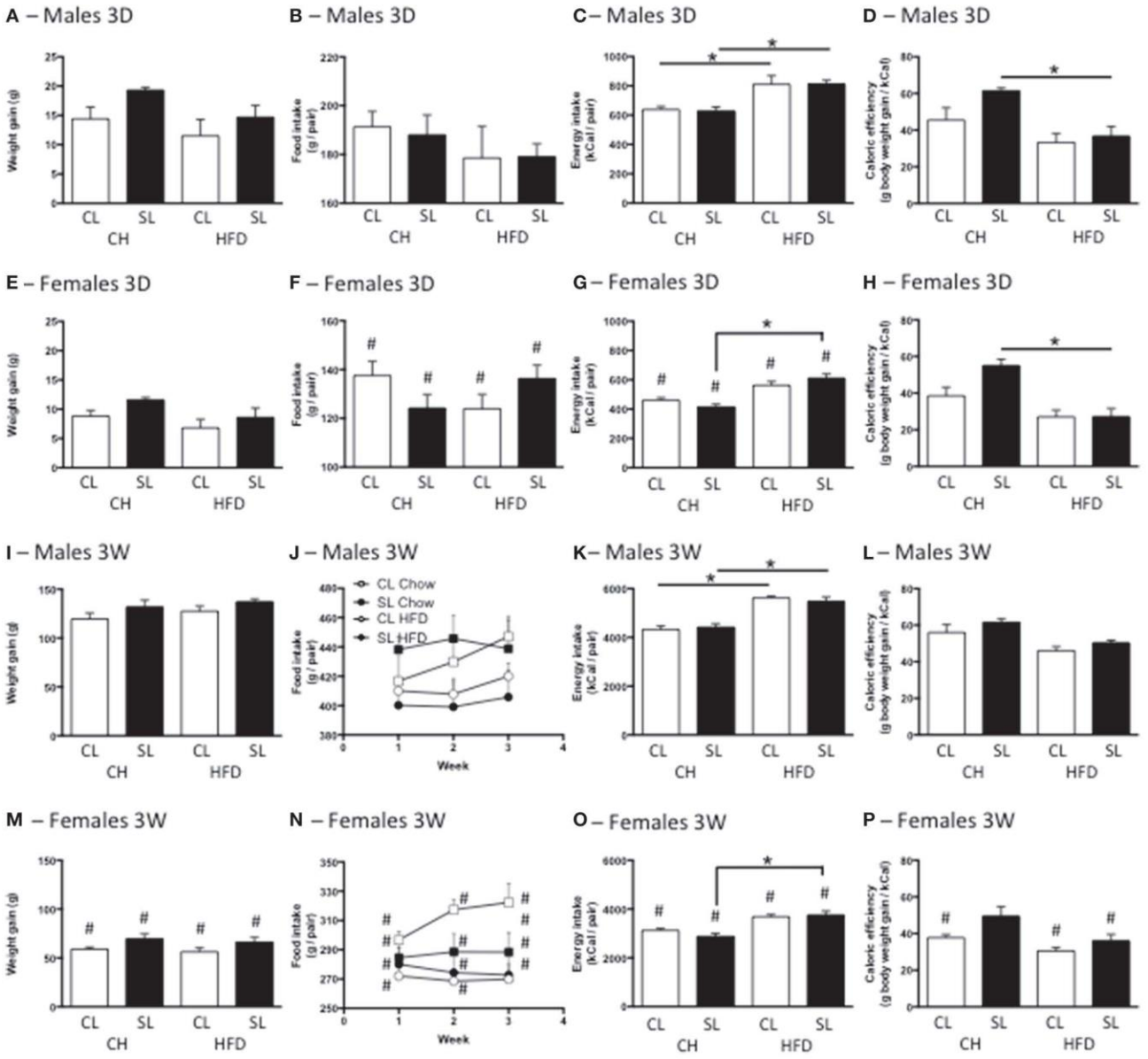

FIGURE 2 | Effects of neonatal overfeeding on weight gain and food intake after 3 day or 3 week high fat diet. (A,E,I,M) Weight gain with 3 day (3D; **A,E**) and 3 week (3W; **I,M**) high fat diet (HFD) or chow (CH) in male **(A,I)** and female **(E,M)** adult rats that were raised in control (CL) and small (SL) litters. 3D HFD: significant effect of sex [$F_{(1,\ 48)} = 26.77$, $P < 0.001$] and diet [$F_{(1,\ 48)} = 7.16$, $P = 0.01$]. 3W HFD: significant effect of sex [$F_{(1,\ 50)} = 374.35$, $P < 0.001$] and litter size [$F_{(1,\ 50)} = 9.78$, $P = 0.003$]. **(B,F,J,N)** Food intake with 3D **(B,F)** and 3W **(J,N)** HFD in male **(B,J)** and female **(F,N)** rats. 3D HFD: significant effect of sex [$F_{(7,\ 48)} = 110.74$, $P < 0.001$]. 3W HFD: significant effect of sex [$F_{(7,\ 46)} = 285.23$, $P < 0.001$] and diet [$F_{(7,\ 46)} = 12.03$, $P = 0.001$]. **(C,G,K,O)** Energy intake with 3D **(C,G)** and 3W **(K,O)** HFG in male **(C,K)** and female **(G,O)** rats. 3D HFD: significant effect of sex [$F_{(7,\ 45)} = 88.54$, $P < 0.001$], and diet [$F_{(7,\ 45)} = 53.58$, $P < 0.001$]. 3W HFD: significant effect of sex [$F_{(7,\ 46)} = 266.19$, $P < 0.001$], and diet [$F_{(7,\ 46)} = 93.34$ $P < 0.001$), significant sex × diet interaction [$F_{(7,\ 46)} = 5.54$, $P = 0.023$]. **(D,H,L,P)** Caloric efficiency with 3D **(D,H)** and 3W **(L,P)** HFD in male **(D,L)** and female **(H,P)** rats. 3D HFD: significant effect of litter size [$F_{(7,\ 45)} = 6.83$, $P = 0.012$], sex [$F_{(7,\ 45)} = 4.53$, $P = 0.039$], and diet [$F_{(7,\ 45)} = 30.73$, $P < 0.001$], significant litter size × diet interaction [$F_{(7,\ 45)} = 4.45$, $P = 0.041$]. 3W HFD: significant effect of litter size [$F_{(7,\ 46)} = 10.75$, $P = 0.002$], sex [$F_{(7,\ 46)} = 51.85$, $P < 0.001$], and diet [$F_{(7,\ 46)} = 25.39$, $P < 0.001$]. Data are mean + SEM. #Sex difference between corresponding groups. *As indicated, $P < 0.05$.

PERIPHERAL INFLAMMATION WITH HIGH FAT DIET AND LPS IN ADULTHOOD; LIVER PROTEIN

Analysis of liver concentrations of a suite of pro- and anti-inflammatory cytokines revealed no notable effects of diet at 3D or 3W in any of the groups, and no notable effects of the litter size except where IL-2 was suppressed in SL relative to CL. LPS significantly increased liver IL-1α, IL-1β, IL-6, and TNFα across the groups, but there were no significant differences

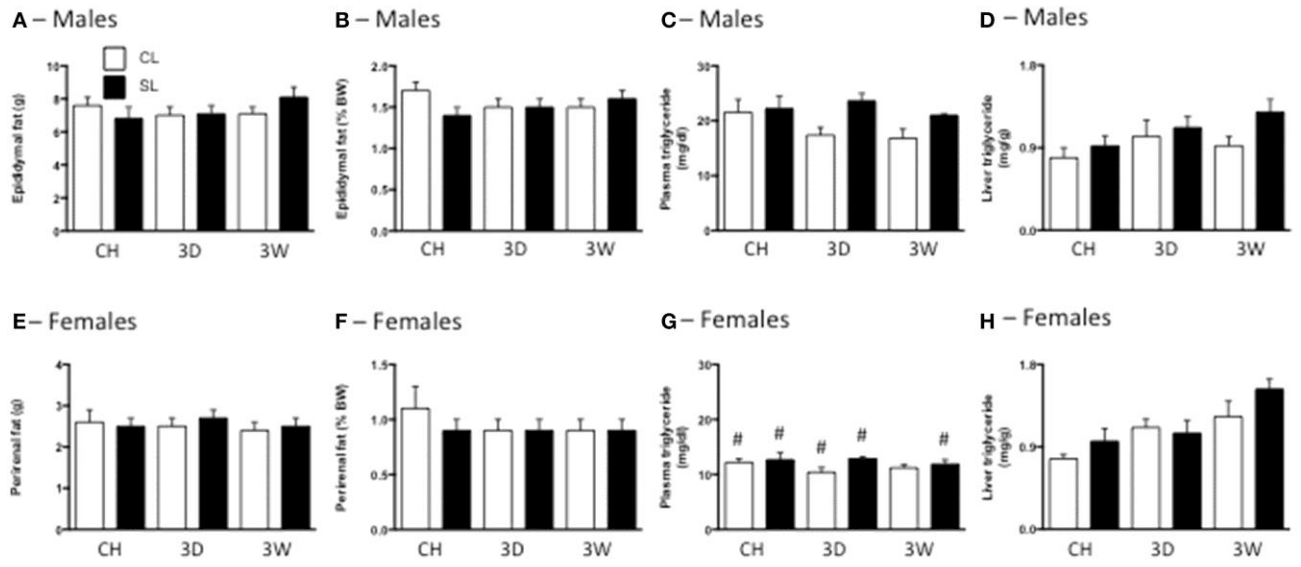

FIGURE 3 | Effects of neonatal overfeeding on fat mass and triglyceride content after 3 day or 3 week high fat diet. Total fat **(A,E)** and percentage fat **(B,F)** after 3 day (3D) and 3 week (3W) high fat diet or chow (CH) in male **(A,B)** and female **(E,F)** adult rats that were raised in control (CL) and small (SL) litters. **(C,G)** Plasma triglycerides in male **(C)** and female **(G)** rats. Significant effect of litter size [$F_{(11,\ 91)} = 4.64$, $P = 0.034$], and sex [$F_{(11,\ 91)} = 81.91$, $P < 0.001$]. **(D,H)** Liver triglycerides in male **(D)** and female **(H)** rats. Significant effect of litter size [$F_{(11,\ 72)} = 4.24$, $P = 0.043$] and diet [$F_{(11,\ 72)} = 5.69$, $P = 0.001$]. Data are mean + SEM. #Sex difference between corresponding groups. $P < 0.05$.

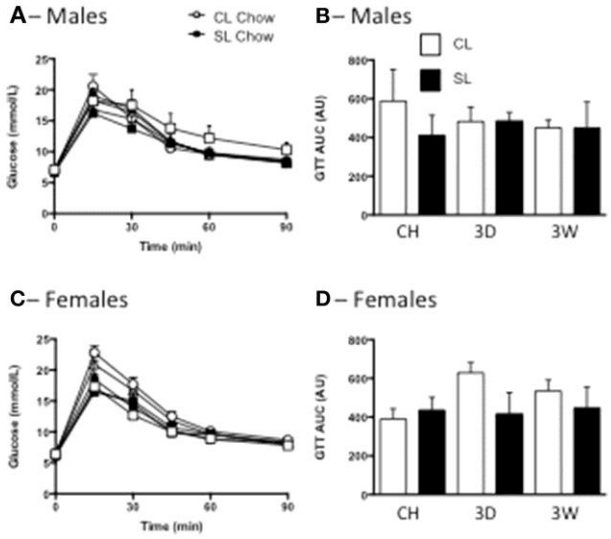

FIGURE 4 | Effects of neonatal overfeeding on glucose utilization after 3 day or 3 week high fat diet. Glucose concentrations **(A,C)** and incremental area under the curve (iAUC) responses **(B,D)** to an i.p. glucose tolerance test after 3 day (3D) and 3 week (3W) high fat diet or chow (CH) in male **(A,B)** and female **(C,D)** adult rats that were raised in control (CL) and small (SL) litters. Data are mean + SEM. There were no differences between groups.

with the *post-hoc* tests except in IL-1α CL after 3D high fat diet. We also found significant sex differences, with less of all the cytokines measured in females than in males except IL-1α (**Table 1**).

MICROGLIAL CHANGES WITH HIGH FAT DIET IN ADULTHOOD

In agreement with our previous findings (Ziko et al., 2014), neonatal overfeeding significantly increased PVN microglial numbers so that under chow-fed conditions, male SL rats had more microglia than CL in this region (**Figures 6A,I**). In males, the 3D high fat diet caused a substantial increase in microglial numbers and density in CL rats but, interestingly, caused a reduction in microglial numbers in SL rats compared with the chow diet (**Figures 6A,I**). After 3W high fat diet, microglial numbers remained elevated in CL compared with under chow conditions, but there was no effect of the 3W diet on SL rats (**Figures 6A,I**). Similar trends were seen in microglial density. In this case, the 3D high fat diet increased microglial density in CL but not SL and the 3W high fat diet had little effect (**Figure 6B**). In females the responses were more ambiguous, with neonatal overfeeding and adult diet having no significant effects (**Figures 6E,F**).

In the ARC there were significant effects of litter size, diet, and sex on microglial numbers, with females having fewer microglia and neonatal overfeeding reducing microglial numbers overall, but there were no relevant differences with *post-hoc* comparisons (**Figures 6C,G**). There was also a significant effect of sex on microglial density in this region with *post-hoc* tests revealing female CL but not SL rats had reduced microglial density compared with males after 3W high fat diet (**Figures 6D,H**).

NEURONAL ACTIVATION WITH HIGH FAT DIET IN ADULTHOOD

As previously demonstrated (Clarke et al., 2012), neonatal overfeeding exacerbates the PVN response to LPS in male rats, with SL males having approximately twice as many mp and mg PVN neurons activated after LPS as CL (**Figures 7A,C**). In male CL rats, the

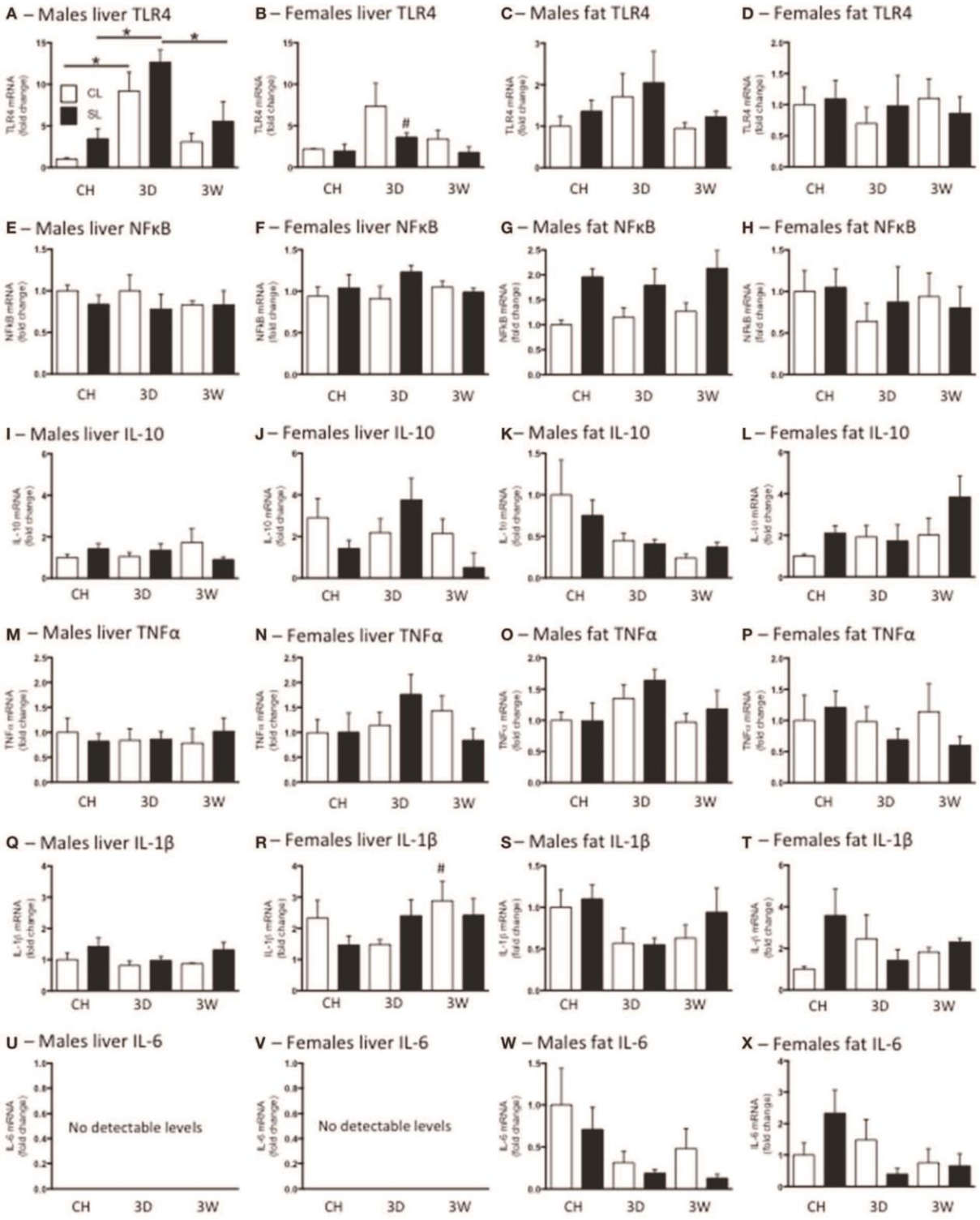

FIGURE 5 | Effects of neonatal overfeeding on peripheral inflammatory gene expression after 3 day or 3 week high fat diet. Liver and fat TLR4 **(A–D)**, NFκB **(E–H)**, interleukin (IL)-10 **(I–L)**, TNFα **(M–P)**, IL-1β **(Q–T)** and IL-6 **(U–X)** after 3 day (3D) and 3 week (3W) high fat diet or chow (CH) in male and female adult rats that were raised in control (CL) and small (SL) litters. Liver TLR4: significant effect of diet [$F_{(11,\ 60)} = 18.71$, $P < 0.001$] and sex [$F_{(11,\ 60)} = 8.25$, $P = 0.006$], significant litter size × sex interaction [$F_{(11,\ 60)} = 7.47$, $P = 0.008$], significant diet x sex interaction [$F_{(11,\ 60)} = 3.39$, $P = 0.04$]. Liver NFκB: significant effect of sex [$F_{(11,\ 56)} = 4.12$, $P = 0.047$]. Male fat NFκB: significant effect of litter size [$F_{(5,\ 33)} = 14.80$, $P = 0.001$]. Liver IL-10 significant effect of sex [$F_{(11,\ 57)} = 11.25$, $P = 0.001$]. Male fat IL-10 significant effect of diet [$F_{(5,\ 30)} = 4.81$, $P = 0.015$]. Liver IL-1β significant effect of sex [$F_{(11,\ 59)} = 26.42$, $P < 0.001$]. Male fat IL-1β significant effect of diet [$F_{(5,\ 30)} = 3.29$, $P = 0.051$]. Data are mean + SEM. #Sex difference between corresponding groups. *As indicated, $P < 0.05$.

Table 1 | Liver cytokine (pg/mL) responses to lipopolysaccharide (LPS) after 3 days (3D) and 3 weeks (3W) in rats that were raised in control (CL) or small (SL) litters.

	CL CH Sal	SL CH Sal	CL 3D Sal	SL 3D Sal	CL 3W Sal	CL 3W Sal	CL CH LPS	SL CH LPS	CL 3D LPS	SL 3D LPS	CL 3W LPS	CL 3W LPS	Main effects
MALES													
IL-1α	58.1 (10)	40.9 (6)	50.9 (5)	44.1 (5)	70.3 (11)	41.5 (6)	247.5 (88)	188.4 (79)	206.9 (60)*	190.7 (75)	186.5 (68)	116.1 (31)	LPS
IL-1 β	807.7 (120.2)	543.5 (66)	682.6 (65)	614.9 (71)	896.1 (132)	626.0 (32)	1533.3 (260)	1453.8 (358)	1559.5 (282)	1487.7 (463)	1643.0 (455)	1114.6 (242)	LPS, SEX
IL-2	174.2 (23)	129.3 (17)	155.4 (14)	132.9 (10)	177.3 (13)	142.2 (6)	164.2 (6)	150.4 (22)	148.8 (13)	151.3 (34)	166.7 (21)	116.8 (15)	LITTER, SEX
IL-4	1018.5 (190)	843.0 (309)	884.5 (297)	682.0 (182)	1040.4 (336)	853.6 (195)	856.6 (232)	1031.4 (341)	721.0 (185)	1163.8 (516)	1081.3 (415)	738.1 (212)	SEX
IL-5	1205.4 (167)	966.9 (185)	1056.8 (223)	905.0 (142)	1163.2 (253)	1034.3 (163)	1007.9 (175)	1151.6 (266)	879.10 (100)	1160.0 (311)	1099.5 (247)	964.1 (181)	SEX
IL-6	84.9 (12)	57.4 (12)	72.7 (10)	56.2 (7)	88.3 (10)	68.8 (11)	106.0 (14)	102.5 (29)	94.2 (18)	109.1 (36)	103.7 (26)	71.4 (10)	LPS, SEX
IL-10	4885.6 (654)	3760.2 (896)	4331.9 (794)	3655.3 (472)	5228.0 (1058)	4326.8 (660)	4764.5 (699)	4952.8 (1105)	4140.0 (567)	5372.0 (1667)#	5021.6 (1108)	3824.8 (680)	SEX
IL-12	341.2 (59)	260.9 (64)	277.0 (84)	224.4 (55)	314.5 (94)	257.9 (61)	263.5 (61)	311.1 (84)	233.2 (49)	316.1 (112)	289.9 (95)	249.0 (61)	SEX
TNF- α	205.7 (31)	157.0 (36)	173.9 (22)	158.8 (19)	221.3 (20)	168.6 (23)	287.2 (47)	232.4 (58)	243.8 (45)	257.9 (67)	284.7 (77)	194.2 (20)	LPS, SEX
FEMALES													
IL-1 α	40.7 (3)	35.6 (5)	44.1 (5)	37.7 (4)	34.5 (2)	68.0 (14)	155.3 (41)	179.0 (28)	292.0 (71)	136.2 (35)	151.8 (26)	167.4 (14)	
IL-1 β	424.1 (54)	324.3 (56)	459.6 (45)	390.7 (35)	377.0 (30)	524.9 (76)	975.4 (172)	1328.1 (227)	1403.6 (241)	930.8 (157)	964.0 (166)	1084.8 (113)	
IL-2	122.1 (7)	93.5 (10)	126.1 (12)	111.6 (11)	107.2 (8)	111.6 (8)	102.9 (14)	100.4 (11)	105.3 (9)	93.5 (13)	106.3 (7)	119.1 (14)	
IL-4	406.5 (56)	313.9 (88)	586.7 (133)	414.1 (116)	369.1 (59)	465.9 (72)	366.1 (96)	287.8 (49)	271.4 (43)	405.7 (83)	350.1 (48)	498.3 (129)	
IL-5	440.0 (36)	399.3 (37)	526.6 (50)	417.1 (37)	423.0 (25)	563.7 (69)	405.4 (58)	412.3 (47)	354.7 (35)	427.3 (59)	397.2 (13)	500.8 (61)	
IL-6	56.0 (7)	41.7 (6)	55.0 (6)	46.3 (5)	42.5 (4)	51.7 (7)	62.8 (8)	68.9 (10)	81.7 (16)	55.1 (9)	55.7 (5)	74.3 (10)	
IL-10	2206.2 (116)	1798.6 (162)	2223.9 (150)	1995.1 (226)	2021.2 (165)	2009.6 (85)	1849.1 (226)	1865.4 (143)	2002.2 (176)	1891.4 (162)	2043.1 (84)	2212.5 (128)	
IL-12	69.8 (7)	50.7 (9)	80.9 (11)	65.5 (8)	61.1 (6)	88.0 (14)	60.7 (11)	67.1 (13)	52.8 (7)	65.8 (11)	60.6 (5)	82.1 (13)	
TNF- α	162.2 (27)	124.4 (19)	129.2 (15)	129.0 (21)	105.1 (13)	133.0 (25)	159.4 (23)	182.2 (32)	203.0 (52)	142.0 (25)	145.9 (13)	182.8 (32)	

*Interleukin (IL)-1α: significant effect of LPS [$F_{(23, 118)} = 70.78$, $P < 0.001$]. IL-1β: significant effect of LPS [$F_{(23, 117)} = 77.74$, $P < 0.001$]; significant effect of sex [$F_{(23, 117)} = 14.28$, $P < 0.001$]. IL-2: significant effect of litter size [$F_{(23, 119)} = 8.47$, $P = 0.004$]; significant effect of sex [$F_{(23, 119)} = 52.93$, $P < 0.001$]. IL-4: significant effect of sex [$F_{(23, 120)} = 34.36$, $P < 0.001$]. IL-5: significant effect of sex [$F_{(23, 120)} = 101.25$, $P < 0.001$]. IL-6: significant effect of LPS [$F_{(23, 120)} = 15.14$, $P < 0.001$]; significant effect of sex [$F_{(23, 120)} = 22.68$, $P < 0.001$]. IL-10: significant effect of sex [$F_{(23, 120)} = 93.59$, $P < 0.001$]. IL-12: significant effect of sex [$F_{(23, 120)} = 95.10$, $P < 0.001$]. Tumor necrosis factor (TNF)α: significant effect of LPS [$F_{(23, 120)} = 14.08$, $P < 0.001$]; significant effect of sex [$F_{(23, 120)} = 20.84$, $P < 0.001$]. Data are mean (SEM). *Versus saline group. #Versus female group. $P < 0.05$.*

3D high fat diet led to a markedly increased PVN response to LPS compared with that seen in chow fed CL rats. This response was not seen in the SL group after 3D high fat diet, where there was a tendency for the PVN response to be reduced compared with chow SL. With 3W high fat diet, neuronal activation after LPS was again similar to that seen in chow-fed rats in both CL and SL, but the difference between these two groups was no longer present.

Interestingly, the females had a different profile of Fos expression in response to LPS (**Figures 7B,D**). Although there were no significant differences between the relevant groups with *post-hoc*

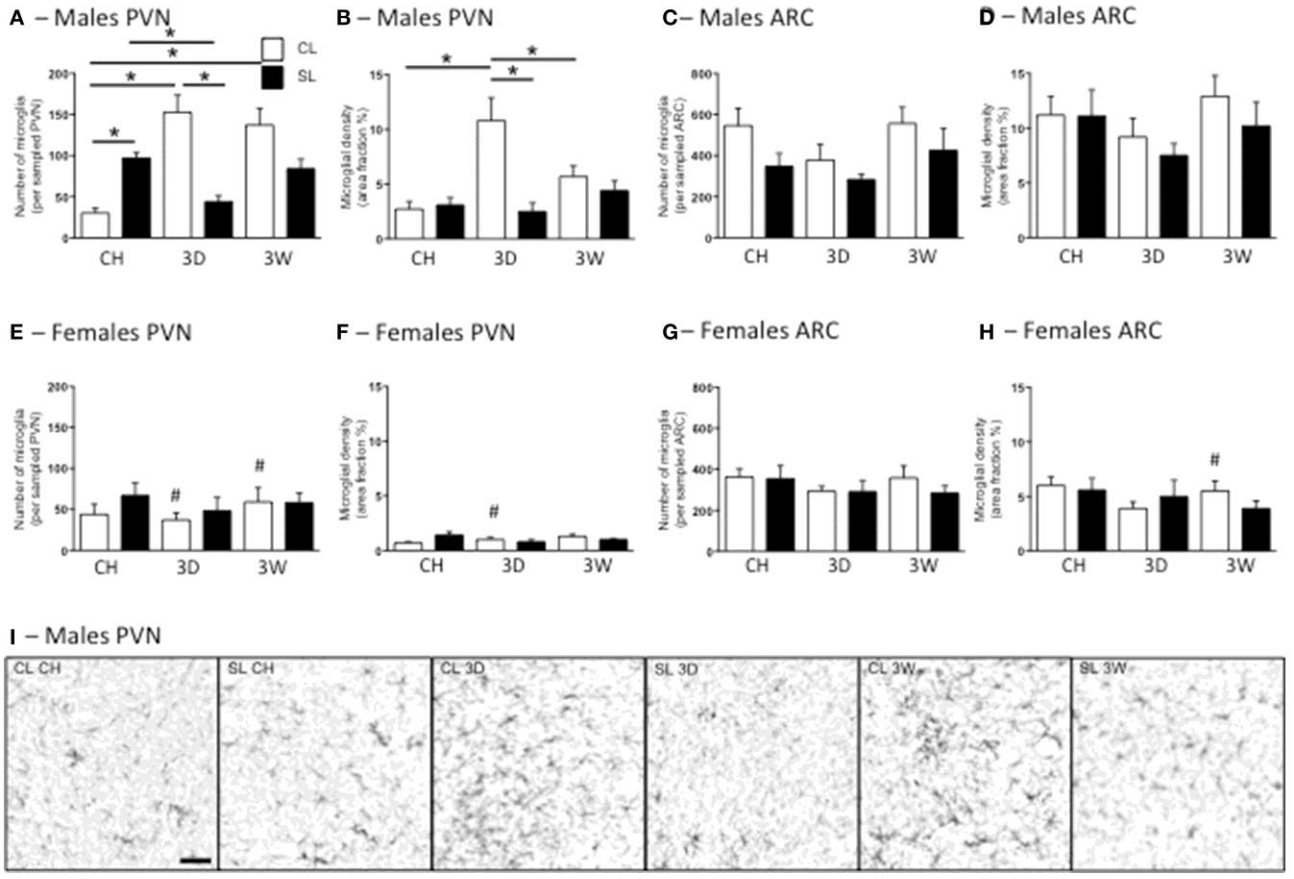

FIGURE 6 | Effects of neonatal overfeeding on hypothalamic microglia after 3 day or 3 week high fat diet. Numbers **(A,C,E,G)** and density **(B,D,F,H)** of ionized calcium-binding adapter molecule-1 (Iba-1)-stained cells after 3 day (3D) and 3 week (3W) high fat diet or chow (CH) in male **(A–D)** and female **(E–H)** adult rats that were raised in control (CL) and small (SL) litters. **(A,B,E,F)** paraventricular nucleus of the hypothalamus (PVN). Microglial number: significant effect of sex [$F_{(11,\ 68)} = 20.53$, $P < 0.001$] and significant litter size × diet × sex interaction [$F_{(11,\ 68)} = 7.06$, $P = 0.002$]. Microglial density: significant effect of litter size [$F_{(11,\ 68)} = 43.68$, $P = 0.029$] and sex [$F_{(11,\ 68)} = 31.96$, $P < 0.001$] and a significant litter size x diet x sex interaction [$F_{(11,\ 68)} = 3.54$, $P = 0.035$]. **(C,D,G,H)** arcuate nucleus (ARC). Microglial number: significant effect of litter size [$F_{(11,\ 68)} = 5.39$, $P = 0.023$] and sex [$F_{(11,\ 68)} = 7.28$, $P = 0.009$]. There was also an effect of diet of $P < 0.06$ [$F_{(11,\ 68)} = 2.95$, $P = 0.059$]. Microglial density: significant effect of sex [$F_{(11,\ 68)} = 38.74$, $P < 0.001$]. Data are mean + SEM. #Sex difference between corresponding groups. *As indicated, $P < 0.05$. **(I)** Representative photomicrographs of the PVN from male CL chow, SL chow, CL 3D, SL 3D, CL 3W, and SL 3W illustrating differences in numbers and density of Iba-1-stained cells. Scale bar = 1 mm.

tests, the trend was for chow-fed SL rats to have a smaller Fos response than CL in both the mp and mg PVN. There was also a trend for the 3W high fat diet to attenuate the response with no effect of the 3D diet. The response to LPS was also significantly higher in males than in females in the CL 3D diet group but, despite an attenuated female response overall compared with males, there were no other significant sex differences with *post-hoc* tests. In the dpPVN there were significant main effects of litter size [$F_{(23,\ 140)} = 14.86$, $P < 0.001$], LPS [$F_{(23,\ 140)} = 13.57$, $P < 0.001$], and sex [$F_{(23,\ 140)} = 49.32$, $P < 0.001$] but no relevant differences with *post-hoc* comparisons (data not shown).

We also examined neuronal activation in several other brain regions involved in fever regulation and the response to LPS. Although the pattern was not as clear as for the PVN, similar responses were also seen in the vBNST and VMPOA in males, with LPS leading to increased Fos in these regions compared with saline after 3D high fat diet in CL but not SL rats (**Figure 7**). Specifically, there was an increase in vBNST Fos in LPS-treated 3D CL males compared with saline-treated 3D CL males, but no other relevant differences. In the dBNST there was an LPS, sex interaction [$F_{(23,\ 126)} = 4.54$, $P = 0.035$], a litter size, sex interaction [$F_{(23,\ 126)} = 4.45$, $P = 0.037$], and a litter size, diet interaction [$F_{(23,\ 126)} = 3.76$, $P = 0.026$], but there were no differences with *post-hoc* tests (data not shown). In the VMPOA there was again a significant increase in Fos in LPS-treated 3D CL males compared with saline-treated 3D CL males, but no other relevant differences. In the MPOA there were effects of LPS [$F_{(23,\ 130)} = 4.48$, $P = 0.036$] and litter size [$F_{(23,\ 130)} = 7.95$, $P = 0.006$], but no differences with *post-hoc* tests (data not shown). In the OVLT there were no relevant differences with *post-hoc* tests except that in females there were more Fos-positive cells with LPS after 3W high fat diet in CL rats than in SL.

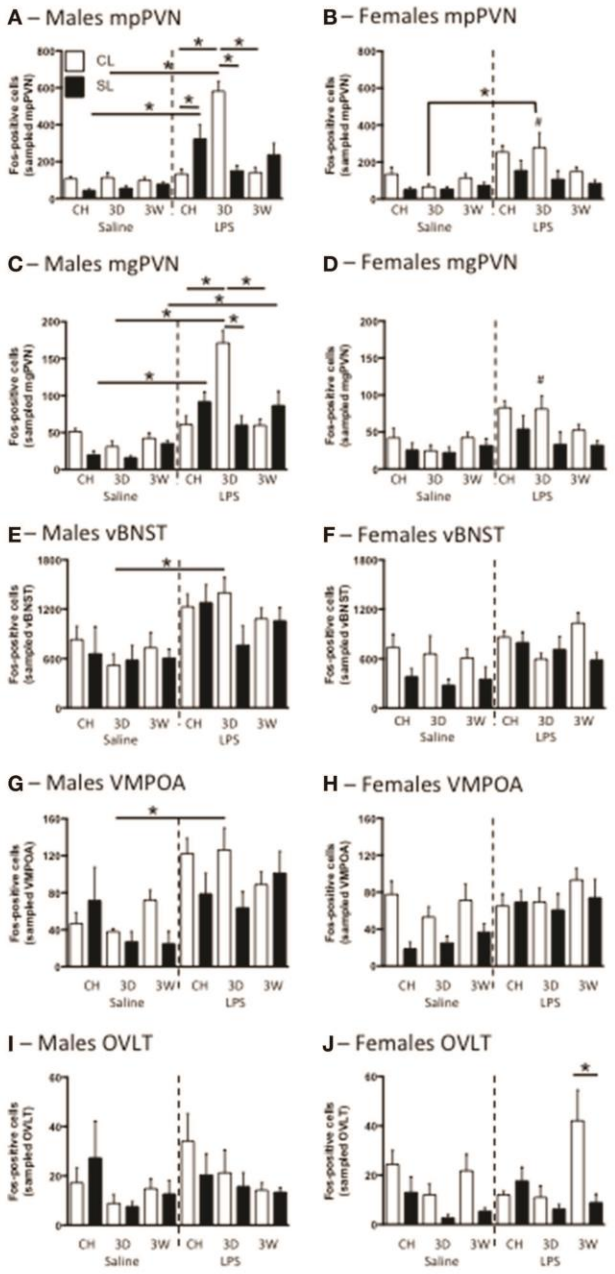

FIGURE 7 | Effects of neonatal overfeeding on neuronal activation in response to LPS. Neuronal activation in the medial parvocellular (mp) **(A,B)** and magnocellular (mg) **(C,D)** paraventricular nucleus of the hypothalamus (PVN), the ventral bed nucleus of the stria terminalis (vBNST) **(E,F)**, the ventromedial preoptic area (VMPOA) **(G,H)** and the vascular organ of the laminar terminalis (OVLT) **(I,J)** with LPS after 3 day (3D) and 3 week (3W) high fat diet or chow (CH) in male **(A,C,E,G,I)** and female **(B,D,F,H,J)** adult rats that were raised in control (CL) and small (SL) litters. mpPVN: significant effect of litter size [$F_{(23,\ 140)} = 15.09$, $P < 0.001$], LPS [$F_{(23,\ 140)} = 68.11$, $P < 0.001$], diet [$F_{(23,\ 140)} = 3.99$, $P = 0.02$], and sex [$F_{(23,\ 140)} = 7.64$, $P = 0.006$] and a significant litter size, LPS, diet, sex interaction [$F_{(23,\ 140)} = 5.22$, $P = 0.007$]. mgPVN: significant effect of litter size [$F_{(23,\ 140)} = 16.85$, $P < 0.001$], LPS [$F_{(23,\ 140)} = 71.26$, $P < 0.001$], and sex [$F_{(23,\ 140)} = 12.35$, $P = 0.001$] and a significant litter size, LPS, diet, sex interaction [$F_{(23,\ 140)} = 3.78$, $P = 0.025$]. vBNST: significant effect of sex

(Continued)

FIGURE 7 | Continued

[$F_{(23,\ 131)} = 15.76$, $P < 0.001$], LPS [$F_{(23,\ 131)} = 31.73$, $P = 0.051$], and litter size [$F_{(23,\ 131)} = 8.05$, $P = 0.005$] and a significant litter size, LPS × diet × sex interaction [$F_{(23,\ 131)} = 3.05$, $P = 0.051$]. VMPOA: significant effect of LPS [$F_{(23,\ 130)} = 31.81$, $P < 0.001$] and litter size [$F_{(23,\ 130)} = 11.70$, $P = 0.001$] as well as a significant litter size x LPS × diet × sex interaction [$F_{(23,\ 130)} = 3.91$, $P = 0.023$]. OVLT: significant effect of diet [$F_{(23,\ 133)} = 5.34$, $P = 0.006$] and litter size [$F_{(23,\ 133)} = 7.64$, $P = 0.007$]. There was also a diet x sex interaction of $P < 0.06$ [$F_{(23,\ 133)} = 2.90$, $P = 0.058$]. Data are mean + SEM. #Sex difference between corresponding groups. *As indicated, $P < 0.05$.

DISCUSSION

The perinatal nutritional environment is important in long-term metabolic programming and, as such, rats that are overfed as neonates by being suckled in small litters show early accelerated weight gain that is maintained into young-adulthood (Spencer and Tilbrook, 2009; Clarke et al., 2012; Stefanidis and Spencer, 2012; Ziko et al., 2014). This model was established as early as the 1960s (McCance, 1962) and these findings have been consistently replicated by several groups (e.g., Plagemann et al., 1992, 2010; Xiao et al., 2007; Chen et al., 2008). Our data now show that despite this weight gain, neonatally overfed rats are only marginally more susceptible to the metabolic/obesigenic effects of a short or medium-term high fat diet. Neonatally overfed rats had an overall increase in plasma and liver triglyceride content on a high fat diet compared with CL rats. They also had a lower caloric efficiency after the high fat diets compared with their chow counterparts, indicating they gained less weight for the same calorie intake, whereas CL rats did not. However, contrary to our hypothesis that SL rats would be more susceptible to the metabolic effects of a high fat diet, they did not eat more, or gain more visceral fat mass, and they did not display early glucose intolerance that might suggest a pre-diabetic profile. They also had no differences in inflammatory gene expression in the liver or fat or in liver cytokine concentrations. We should note that different fat pads can respond differently to dietary influences and a comprehensive analysis of the different depots may still reveal differences between the groups. However, we did not find differences in any other metabolic or inflammatory parameters to suggest this is a strong possibility.

Interestingly, these neonatally overfed rats did show differences in susceptibility to the central pro-inflammatory effects of short-term (3 days) high fat feeding. Thus, 3 days high fat diet led to significant microgliosis in the PVN in male CL rats, but this was not seen in SL. This increase in microglial numbers in the PVN was still evident at the 3 week mark. Similarly, the PVN response to LPS was markedly enhanced in CL rats by 3 days of high fat diet, but not in SL, although this response was resolved to control levels at 3 weeks. These differences were not seen in the females.

In a recent study, Thaler and colleagues have suggested the early (3 day) inflammatory response to a high fat diet is actually an adaptive one and that it is only with longer-term (e.g., 3 weeks) high fat feeding that a maladaptive pro-inflammatory response ensues (Thaler et al., 2012). Thus, at 3 days on a high fat diet, rats and mice in the Thaler study showed hypothalamic microgliosis and an increase in hypothalamic pro-inflammatory

gene expression. This profile disappeared by 7 days but returned after 21 days of high fat diet (Thaler et al., 2012). In light of this work, our findings would suggest that the absence of microgliosis or an exacerbated response to LPS after 3 days of high fat diet in SL is maladaptive; reflective of an inability to effectively respond to the high fat diet. However, if this interpretation is correct, one would expect to see differences between the groups at 3 weeks, which we did not see in this study.

In this investigation, we deliberately selected relatively short periods of high fat feeding. Our hypothesis suggested neonatally overfed rats may be more susceptible to a high fat diet and it was therefore essential to give a metabolic challenge mild enough to avoid a ceiling effect. As with the present study, other groups have seen 3 weeks of high fat diet is not usually sufficient to induce overt body weight and fat mass differences. For example, Maric and colleagues have shown 32% calorie by fat diets for 8 weeks do not cause a difference, compared with chow fed, in fat pad weight in Wistar rats and only cause a significant increase in total weight gain if the diet is butter based (and not if it is coconut oil-based) (Maric et al., 2014). Significant metabolic and inflammatory effects of both a 3 day and 3 week high fat diet have been reported (Thaler et al., 2012), suggesting the dietary challenge in this study would be sufficient to induce inflammation and allow us to detect any differences between the neonatally overfed and control rats. However, it is possible that while an initial adaptive response to the high fat diet was evident at 3 days, 3 weeks was insufficient to reveal susceptibility to the metabolic effects of the challenge. If this is the case, we would expect the neonatally overfed rats to respond differently after a longer period of high fat diet. It is interesting that our 3 day high fat diet actually caused an overall reduction in the normal weight increase. This is likely to be related to the novelty of the new diet, since other experimental factors would also have influenced the chow groups. What this means for the inflammatory outcome is unclear, especially since the elevated energy intake at 3 days in the high fat diet-fed groups implies they were consuming the high fat chow as expected and any reductions in weight gain may therefore be due to non-nutrient factors. It is possible an adaptive anti-inflammatory response to acute high fat diet (Thaler et al., 2012) is aided in rodents by food-novelty-related elevations in glucocorticoids, but this possibility remains to be tested.

One of the more interesting findings to come out of the present study is our evidence of central pro-inflammatory changes in the absence of a significant change in the metabolic or peripheral pro-inflammatory profiles. Apart from an increase in liver TLR4 mRNA in both CL and SL groups at 3 days high fat diet, there were no significant changes in peripheral indicators of obesity or inflammation in the tissues we examined. These data support recently published evidence (Thaler et al., 2012; Maric et al., 2014). Although it has long been recognized obesity is associated with peripheral inflammation, including elevated pro-inflammatory cytokines in circulation (Hotamisligil et al., 1995; Hotamisligil, 2006), more recent evidence, and our own from this study, is suggesting central inflammation and neuronal injury with high fat diet actually precedes peripheral inflammation. The systemic inflammatory response to diet or weight gain is derived from excess macrophage infiltration to the adipose tissue and subsequent excess production of pro-inflammatory cytokines. It is likely this is a relatively chronic process and possible it is driven, to a degree, by central inflammation (Weisberg et al., 2003; Xu et al., 2003). Thaler and colleagues have shown markers of inflammation in the hypothalamus are elevated as early as 24 h after the onset of a high fat diet. Within a week this is reflected in neuronal injury. Indices of peripheral inflammation, however, are not evident until weeks to months of the diet (Thaler et al., 2012). Similarly, Maric and colleagues have shown diet high in saturated fat leads to central inflammation in the absence of peripheral even as late as 8 weeks after onset (Maric et al., 2014). Our current findings tend to support these suggestions that central inflammation, at least in terms of microgliosis and susceptibility to an immune challenge, occurs early after the commencement of a high fat diet, and precedes the development of metabolic dysregulation or an obese profile. In this regard, it will be interesting to examine how high fat diet influences acute pro-inflammatory circulating signals such as leptin in these neonatally overfed populations, since adipokines such as leptin are important in influencing central inflammation (Gao et al., 2014).

Our findings also suggest that a short period of high fat diet feeding may actually leave the individual seriously vulnerable to bacterial infection at this time. A hypersensitive HPA axis after 3 days high fat diet may be an adaptive attempt to curtail inflammation through glucocorticoid production (Thaler et al., 2012). However, our CL rats given 3 days high fat diet responded to LPS with a six-fold increase in neuronal activation in the PVN. Although we did not see differences from the chow-fed groups in LPS/fever-regulatory brain regions (VMPOA, OVLT, BNST) and we did not measure fever and sickness behavior directly, a response of this magnitude in the PVN is likely to reflect a more severe illness with LPS (Tarr et al., 2012). Several studies have shown microglia behave differently depending upon their background or basal state. For instance, early life immune challenge can leave microglia "primed" to more readily respond to a similar challenge later on (Bland et al., 2010; Williamson et al., 2011). We have recently shown neonatal overfeeding has a similar effect, with neonatally overfed rats having an exaggerated microglial, febrile, cytokine, and HPA axis response to LPS (Clarke et al., 2012; Ziko et al., 2014). The present work suggests that instead of exacerbating this response, the 3 day and 3 week high fat diets dampen it, at least in terms of PVN neuronal activation after LPS, uncovering the possibility of an interaction between the "primed" microglial state and subsequent diet.

Another notable finding of the present study is that the sexes responded quite differently to the high fat diet. While CL males were affected by 3 days high fat diet in a number of parameters, females were not. We deliberately did not control for cycle stage in our females as this imposes an additional stressor on the animals. However, we believe cycle stage is unlikely to account for these sex differences since the variability in the data was similar for females as for males. Although few investigators have examined both males and females in the same study, our findings do concur with reported literature. For instance, male mice develop insulin resistance after a short period of diet high in saturated or unsaturated fat. Female mice retain their insulin sensitivity with the same diet (Senthil Kumar et al., 2014). Likewise, female rats

are relatively protected against the metabolic effects of a high fructose or sucrose diet, whereas males develop insulin resistance and hypertension under the same conditions (Galipeau et al., 2002). Our data thus illustrate female rats are likely to be more resilient to the effects of short-term high fat diet than males. These data also highlight the importance of including both sexes as study subjects, or at least exercising care when extrapolating data from one sex to another.

In summary, rats made overweight by early life overfeeding are unlikely to be substantially more vulnerable to a short-term adult-onset high fat diet than control rats in terms of developing further obesity or a diabetogenic profile. On the other hand, neonatally overfed rats were less responsive to the central pro-inflammatory effects of a 3 day high fat diet than controls. Whether this represents a maladaptive inability to combat the central effects of the high fat diet or, rather, a resilience to the challenge, remains to be determined in future work.

AUTHOR CONTRIBUTIONS

Guohui Cai, Juan Molero and Sarah J. Spencer conceived of and designed this study. Guohui Cai, Ilvana Ziko, Stanley M. H. Chan, Xiao-Yi Zeng, Songpei Li, Juan Molero, and Sarah J. Spencer ran the animal studies and collected samples. Guohui Cai, Tara Dinan, Joanne M. Barwood, Simone N. De Luca, Alita Soch, and Sarah J. Spencer analyzed samples and interpreted the data. Sarah J. Spencer wrote the manuscript. All authors revised the manuscript critically. All authors give final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

Appendix 3 RMIT University Animal Ethics Committee approval letter

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04 July 2013

Dr Sarah Spencer
School of Health Sciences
RMIT University

Dear Sarah,

AEC Project No. 1229: Developmental programming of adult stress responses: how early life nutrition permanently alters stress in rats – Amendment 1

I am pleased to advise that the requested amendment for the above project was approved by the RMIT University Animal Ethics Committee (AEC). An approved version of the amendment is attached.

This amendment was to add 2 new staff members (Ms Ilvana Ziko and Mr Jason Nguyen) and to make changes to the experimental design.

On behalf of the AEC I wish you well with your research.

Yours sincerely

Leia Demtschyna
Coordinator Animal Ethics and Gene Modification
On behalf of
RMIT Animal Ethics Committee

cc: Ms Tricia Murphy, RAF manager

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04 July 2013

Dr Sarah Spencer
School of Health Sciences
RMIT University

Dear Sarah,

AEC Project No. 1230: Developmental programming of adult stress responses: how early life nutrition permanently alters immune function in rats – Amendment 2

I am pleased to advise that the requested amendment for the above project was approved by the RMIT University Animal Ethics Committee (AEC). An approved version of the amendment is attached.

This amendment was to add 1 new staff member (Ms Ilvana Ziko) and to make a change to the experimental design.

On behalf of the AEC I wish you well with your research.

Yours sincerely

Leia Demtschyna
Coordinator Animal Ethics and Gene Modification
On behalf of
RMIT Animal Ethics Committee

cc: Ms Tricia Murphy, RAF manager

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4 July 2013

Dr Sarah Spencer
School of Health Sciences
RMIT University

Dear Sarah,

AEC Project No. 1337: Developmental programming of adult predisposition to obesity in the rat

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from **1 August 2013** until **1 August 2016**. An approved version of the application is attached.

The use of animals in scientific procedures is strictly regulated by the *Australian code of practice for the care and use of animals for scientific purposes* (the 'Code'). The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare. There are several aspects of the Code, the license conditions and the operations of the AEC that I would like bring to your attention.

Responsibilities of investigators

Responsibilities of investigators are described in the *Australian code of practice for the care and use of animals for scientific purposes* (section 3). According to the Code investigators have a 'personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the Code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project' (s.3.1.1).

Amendments and extensions

If as you proceed with your project you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the Committee may only deal with 'minor' amendment requests. Major amendments to projects normally require a new project application.

Minor amendments including the addition of staff to a project or requests for time extensions can be reviewed by an executive between meetings if necessary, otherwise requests are dealt with at the regular scheduled meetings of the AEC. If executive approval is required please contact the secretary directly.

Time extensions are normally granted for projects, but cannot be granted retrospectively in any circumstances.

Adverse events or unexpected outcomes

As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the Committee immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.

Animals that are ill or unwell need to be reported immediately via the care forms available at the RMIT Animal Facility. Dr Shy Seah is the Animal Welfare Officer at RMIT and should be contacted as well in the case of any emergency. She can be contacted on **0409 521 234** at any time. In case of any unexpected animal death please remember that the researcher has a responsibility to organise an autopsy so as to determine the cause of death.

Investigator guidelines for record keeping
I draw your attention to the document, 'Investigator guidelines for record keeping', which was prepared by the Bureau of Animal Welfare, which is available on the AEC website. Investigators are required to adhere to the strict guidelines regarding record keeping for their project. Note that records associated with a project 'should be available for audit by the institution and authorised external reviewers'. Failure to maintain proper records may result in a compliance breach of the Code and place at risk the researcher's capacity to carry out research with animals.

Conditions of approval
The Animal Ethics Committee may apply conditions of approval beyond the submission of annual/final reports. There are no specific conditions attached to this project, except that described elsewhere in this letter.

Reports
Approval to continue a project is conditional on the submission of annual and final reports. Annual reports are requested in December each year, and must be submitted whether or not the project has commenced or is inactive. Report forms are available from the Animal Ethics Committee web site.

Please note that failure to submit reports will mean that a project is no longer approved, and/or that approval will be withheld from future projects.

All reports or communication regarding this project are to be forwarded to the secretary.

Copies of forms and information referred to above are available from the AEC website:
http://www.rmit.edu.au/staff/research/research-integrity-and-governance/animal-ethics

On behalf of the AEC I wish you well with your research.

Yours sincerely

Leia Demtschyna
Coordinator Animal Ethics and Gene Modification

cc: Ms Tricia Murphy, RAF manager and Dr Shy Seah, Animal Welfare Officer

RMIT UNIVERSITY

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